ANNUAL REPORTS IN MEDICINAL CHEMISTRY
Volume 12

Sponsored by the Division of Medicinal Chemistry of the American Chemical Society

Editor-in-Chief: **FRANK H. CLARKE**

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Volume 12

CONTRIBUTORS

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PREFACE

Annual Reports continues to provide timely reviews on traditional aspects of medicinal chemistry and seeks to describe new approaches to medicinal research which are likely to have increasing importance for drug discovery and development. Many of the chapters discuss the mechanisms of action of drugs and present concepts of the nature of disease which may assist in the design of agents for its control.

The importance of drug metabolism for medicinal chemists is emphasized this year with one chapter describing its use in drug discovery and development and a second chapter on the use of stable isotopes in drug metabolism. A chapter on animal models for memory and learning augments the more traditional CNS topics. Cerebral vasodilators are described this year in the section on pharmacodynamic agents. Other new topics include discussion of the biosynthesis of antibiotics, psoriasis, adenylate cyclases, biochemical procedures for syntheses and computer assisted syntheses. As expressed in the section editorial in Topics in Biology, chapters are included which describe pathophysiology at the molecular and biochemical level. Of special interest is the chapter on tests for the detection of mutagens and the correlation between mutagenicity and carcinogenicity of chemicals.

The Editors of Annual Reports in Medicinal Chemistry continue to welcome comments from its readers. We are especially interested in suggestions for new topics which will make future volumes more useful.

Ardsley, New York
June, 1977

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Section I - CNS Agents

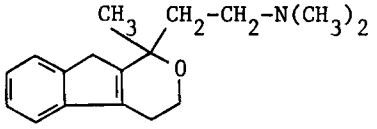
Editor: John Krapcho, Squibb Institute, Princeton, N.J.

Chapter 1. Antidepressants and Antipsychotic Agents

Robert A. Lahti, The Upjohn Company, Kalamazoo, Michigan

Introduction - This review article covers the pharmacological, biochemical and clinical advances during the past year in the area of antidepressant and antipsychotic agents. Past issues of this series [1,2] as well as reviews of greater depth are recommended to the reader. [3-5]

Antidepressant Agents - The past year has produced a number of agents which block either serotonin (5-HT) or norepinephrine (NE) uptake selectively. This direction is an extension of the interest in subclasses of depression based on altered excretion rates of indoles or catechols in depressed patients.[6] Pirandamine (1), an indenopyran, has been shown to be a very selective blocker of 5-HT uptake[7] using the blockade of brain 5-HT depletion by H75/12 (4-methyl-alpha-ethyl-m-tyramine). A closely related compound, tandamine (2), which is a thiopyranoindole, is a strong blocker of NE uptake but has no effect on 5-HT uptake[8] and only weakly potentiates the effects of 5-HTP.[9]



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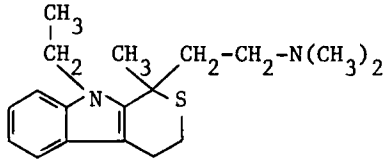
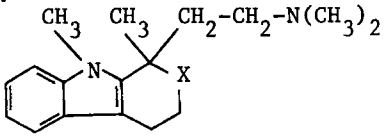


2

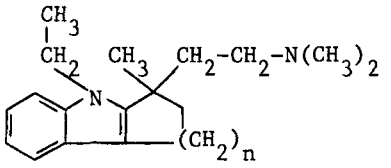
Synthesis of the tandamine analog (3) containing an N-methyl group produced a compound with strong NE uptake blocking activity as well as a strong potentiator of 5-HTP.[9] A pyranoindole analog (4) of pirandamine was found to be a strong blocker of NE uptake, in contrast to pirandamine, but retained the 5-HTP potentiating activity.[9] Interestingly, the thiopyranoindole (3) and the pyranoindole (4) had very similar pharmacological activity.



3 X = S
4 X = O

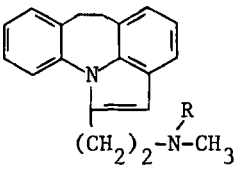
Other variations of tandamine (2) in the form of cycloalkylindoles proved to be of considerable interest.[10] The structurally closest analog (5) to tandamine was very potent in reversing reserpine ptosis, while the cycloheptyl (6) and cyclopentyl (7) derivatives were progressively weaker

in reversing reserpine-induced ptosis. All three of these compounds were more potent than imipramine or amitriptyline in reversing ptosis.

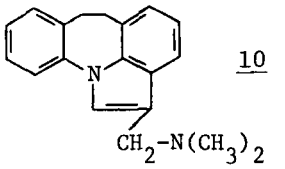


5 n = 2
6 n = 3
7 n = 1

A number of variations of a tricyclic indole structure were prepared. Compound (8) was comparable in activity to imipramine in reversing ptosis and anticholinergic activity. Compound (9), which has the terminal N-benzyl function, was active in reversing reserpine ptosis and showed no anticholinergic and antihistaminic activity. Compound (10) was significant in that it demonstrated the importance of the location of the side chain, this form being inactive. [11]

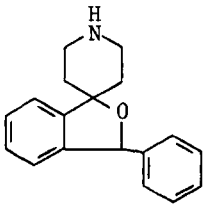


8 R = CH_3
9 R = CH_2- 



10

The spiroisobenzofuran piperidine derivative (11) has potency greater than imipramine in reversing tetrabenazine ptosis. [12]



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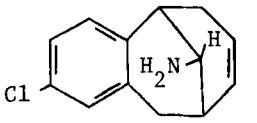
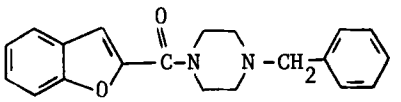


12

Org 6582 (12) was found to be a strong, long lasting blocker of 5-HT uptake, five times chlorimipramine, with no apparent effect on NE uptake systems. [13] Compound 12 also caused a decrease in 5-HT turnover and lowered brain 5-HIAA (5-hydroxyindole acetic acid).

Deviations from the standard tricyclic structures were frequent during this period as demonstrated by DIV-154 (13) which antagonized reserpine, potentiated the effects of NE, and had little anticholinergic activity. [14] Deximafen (14) was reported to be a potent reserpine and tetrabenazine antagonist with little overt activity of its own. [15]



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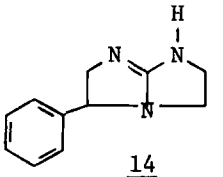
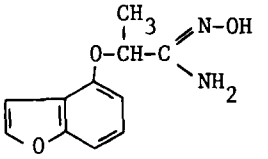


14

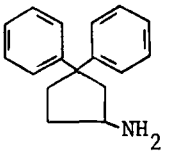
A series of benzofuranyl amidoximes was found to inhibit reserpine ptosis, potentiate yohimbine toxicity, and potentiate the effects of amphetamine.[16] Compound (15) appeared to be the most interesting structure.

15



Of a number of phenylcycloalkylamines, compounds (16) and (17) had similar activity in blocking NE and 5-HT uptake and in potentiating the behavioral effects of DOPA (3,4-dihydroxyphenylalanine) and 5-HTP (5-hydroxytryptophan). However, compound (18), a cyclohexylamine, was inactive except for DOPA potentiation and the cyclohexeneylamine (19) retained the activity of the cyclopentane analogs (16) and (17).[17]

16



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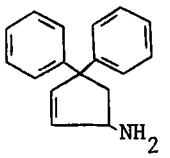

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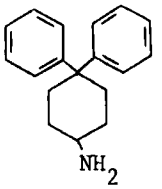

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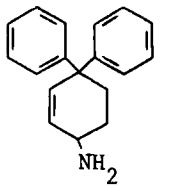
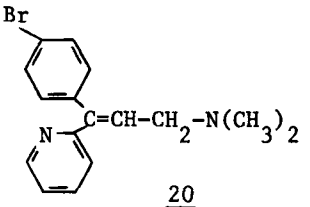


H-102-09 (20) was tested in parallel with chlorimipramine for its 5-HT uptake blocking activity as measured by observing decreases in 5-HT in whole blood.[18] Compound 20 was more potent than chlorimipramine in reducing blood 5-HT and initial clinical studies suggest good antidepressant activity. The antianxiety agent, trazodone (21), has also been found to be a blocker of 5-HT uptake into rat brain synaptosomes.[19] The activity of trazodone was slightly less than that of chlorimipramine, but it had greater selectivity when NE uptake inhibition was determined.



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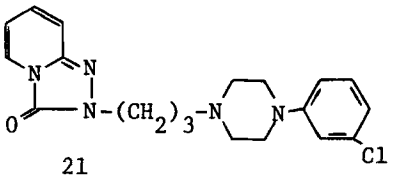
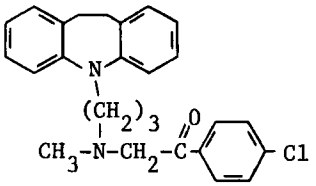


21

A drug metabolism study in man [20] showed that lofepramine (22) was rapidly converted to desmethylimipramine, which probably accounts for the observed blockade of NE uptake.



22

Numerous studies have attempted to define biochemical parameters which would assist in selecting the method of treatment of depression. Subgroups of depression have been defined by urinary MHPG [21] (3-methoxy-4-hydroxyphenylglycol) excretion rates and by the levels of biogenic amines or their metabolites in CSF [22] (cerebrospinal fluid). Low urinary MHPG excretors respond well to imipramine, desmethylimipramine and nortriptyline, whereas high MHPG excretors respond well to amitriptyline. [6,23] Patients with low CSF 5-HIAA respond well to chlorimipramine or 5-HTP but not to nortriptyline.

It has been shown that some unipolar depressed female patients have high COMT (catechol-O-methyl transferase) activity in their red blood cells, and that this observation correlates with a poor response to imipramine. [24] On the other hand, high COMT activity may correspond to high MHPG excretion. This finding supports the previous disclosure that low MHPG excretors respond to imipramine.

During the past year several studies have appeared in the literature bearing on the relationship of 5-HT and/or its precursor to depression. Shopsin et al., in two studies, were able to show that the positive therapeutic response of depressed patients to imipramine [25] or tranylcyromine [26] could be reversed by co-administration of the tryptophan hydroxylase inhibitor PCPA (p-chlorophenylalanine). These same investigators could not show any effect of alpha-methyltyrosine, a tyrosine hydroxylase inhibitor, on the positive clinical response to imipramine. [25] These data seem to indicate that serotonin, rather than catecholamines, is involved in depression.

It was reported [27] that lithium, ECT and tricyclic antidepressants were all associated with a decreased accumulation of 5-HIAA in CSF of probenecid-treated patients, suggesting that antidepressant therapy may alter

5-HT turnover. Others [28] have shown that chlorimipramine lowers CSF 5-HIAA in depressed patients, and it was reported [29] that chlorimipramine, amitriptyline, and desipramine all reduce blood tryptophan levels. Earlier it had been mentioned that chlorimipramine lowered whole blood 5-HT. [18] It has also been shown that chlorimipramine and tryptophan were better in treating depression than chlorimipramine alone, [28] however elsewhere [29] it was reported that no significant difference between any of the following treatment modalities was found: chlorimipramine, chlorimipramine plus tryptophan, desipramine plus tryptophan, and chlorimipramine plus desipramine.

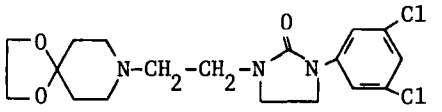
Investigators [30] found no distinct relationship between either total plasma tryptophan or tyrosine and depression. However, they [30] did report that free plasma tryptophan in depressed patients was always higher than controls and returned to normal upon good clinical improvement. In contrast, other investigators [29,31] found lower plasma tryptophan levels in depressed patients than they did in controls.

Utilizing platelets from depressed patients, it was shown [32] that a significant reduction in mean 5-HT and DA uptake occurred in endogenously depressed patients but not in neurotic depressives. In this same study [32] it was reported that chlorimipramine caused a rapid reduction in 5-HT uptake, whereas protriptyline administration had no effect.

Tuomisto and Tukiainen [33] studied the platelet 5-HT uptake system in a well-defined study and found that 5-HT uptake in platelets from depressed patients had one-half the initial uptake value and Vmax of normals; Kms were the same for both groups. Following antidepressant treatment, the Vmax moved towards normal, and the Km remained the same. These results were interpreted to mean that there are less 5-HT transport molecules or some inactive ones in membranes of depressed patients, but those 5-HT transport molecules which are there are completely functional, as demonstrated by the constant Km. Clinical improvement was accompanied by a return of Vmax to normal. Of most interest was the finding that imipramine added *in vitro* to the platelets increased the Km but had no effect on the Vmax.

In summary, the numerous clinical studies cited here and reviewed elsewhere [34] do little to strengthen the catecholamine hypothesis of depression. The profusion of data relating tryptaminergic systems to depression [31-33] is most interesting and hopefully reflects progress in this area. The abundance of new compounds which are selective NE or 5-HT uptake blockers should do much to confirm or deny the role of biogenic amines in depression and the significance of blocking amine uptake as a mode of action of antidepressants.

Antipsychotic Agents - In acute schizophrenia patients, the potent, long-lasting tranquilizer, AL 1965 (23) showed neuroleptic activity and a high incidence of extrapyramidal side effects (EPS). [35]



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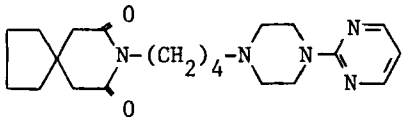
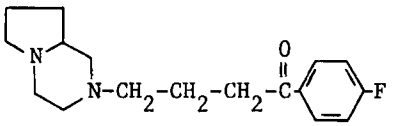
In acute schizophrenic patients, MJ9022 (24) was a weak, short-lasting neuroleptic.[36] Clinical activity correlated with the blocking of amphetamine stereotypy in dogs.



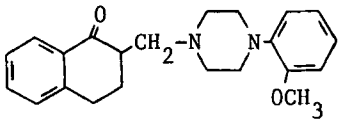
24

A simple butyrophenone, azabuperone (25), was found to produce catalepsy, give a positive response in conditioned avoidance responding, and block amphetamine stereotyped behavior in rats. The compound is currently being used clinically as an antipsychotic agent.[37]



25

A series of novel neuroleptic agents derived from alpha-tetralone and N-arylpiperazine has been prepared.[38] Moderate activity in inhibiting amphetamine hypermobility and in producing hypothermia was found, with compound (26) being the most active. Another N-arylpiperazine analog (27) also had neuroleptic activity in antagonizing amphetamine and apomorphine stereotypy and production of catalepsy.[39]



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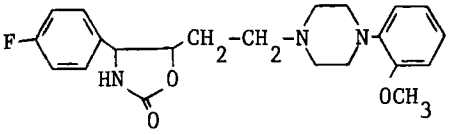
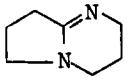
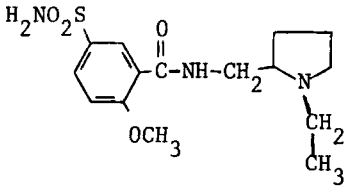


27

DBN (28) is an inhibitor of indoleamine-N-methyltransferase and effectively blocks the synthesis of N,N'-dimethyltryptamine (DMT) *in vitro* and *in vivo*.[40] DMT has been considered as an endogenous psychotogenic agent.[41] Therefore, based on a rational approach to drug therapy, compound 28 may be an antipsychotic agent.[40]

28 

29 

Sulpiride (29) has been tested as an antipsychotic agent[42,43] and is atypical in its pharmacological profile. Sulpiride does not block DA-stimulated adenylate cyclase either in vitro or in vivo,[44] it does not produce catalepsy[45] nor antagonize amphetamine.[45] Sulpiride does block apomorphine-induced emesis in dogs[44] but only weakly antagonizes other effects of apomorphine,[46] yet it causes an increased turnover of DA in vivo.[45]

The ^{3}H-haloperidol binding assay[47,48] has proven to be a most effective in vitro test system for detecting neuroleptic agents (see Chapter 25). Results from this test correlate well with human clinical doses,[47,48] inhibition of apomorphine stereotypy,[47] inhibition of apomorphine-induced emesis in dogs[47] and inhibition of amphetamine stereotyped behavior in rats.[47] ^{3}H-Haloperidol binding data did not correlate well with inhibition of DA-stimulated adenylate cyclase[49] nor with the inhibition of ^{3}H-DA binding.[47,48] The lack of correlation between inhibition of ^{3}H-haloperidol and ^{3}H-dopamine binding may be indicative of a two-state model of the receptor[49] or it may indicate that the two receptors are not the same.[48] The good correlations between most neuroleptic test methods and human clinical dose may reflect the strength of the DA hypothesis[50] or the degree to which the hypothesis has not been tested.

Although much evidence supports the DA hypothesis of schizophrenia, there have been some viable concerns regarding the theory, such as: the clinical efficacy of clozapine as related by Burki et al.,[51] the time dependent effect of neuroleptics on dopamine turnover in psychiatric patients[52] and the lack of correlation between results obtained with neuroleptic agents in the ^{3}H-haloperidol and ^{3}H-dopamine binding systems.[47,48] Also, the dopamine blockade hypothesis may explain the effectiveness of the neuroleptic drugs, however, there is little evidence to support DA overactivity in schizophrenia.[53]

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Chapter 2 Anti-Anxiety Agents, Anticonvulsants and Sedative-Hypnotics

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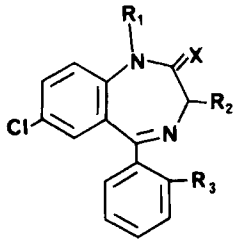
Benzodiazepines and Related Compounds

Studies in cats, mice and rats failed to support the interaction of benzodiazepines and mammalian central glycine receptors which had been proposed on the basis of in vitro studies of strychnine binding.[1,2] The binding of 11 benzodiazepines to bovine serum albumin (BSA) revealed that most have 2—3 binding sites on BSA in contrast to a single site in human SA.[3]

Metabolic studies of lorazepam[4] (1a) in man and animals, pinazepam[5] (2a) in man, diazepam (2b) in human kidney cortex microsomes[6] and bile,[7] and nitrazepam (2c) in rabbit urine[8] have been reported. N-Desmethyldiazepam (2d) is a new human metabolite of chlorodiazepoxide.[9] Diazepam and its main active metabolite desmethyldiazepam pass into the breast milk of nursing mothers during repeated oral administration.[10] Pharmacokinetic studies of lorazepam[11] and chlorazepate[12] (1b) in humans and diazepam in humans and animals[13] have appeared.

Lorazepam (1a), diazepam and chlorodiazepoxide seem to induce some amnesia in rats as evidenced by learning or noxious events and extinction.[14]



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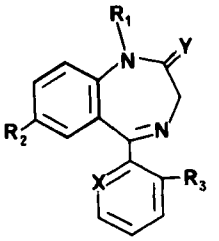

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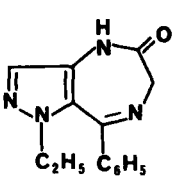
	X	Y	R_1	R_2	R_3
1a	O	-	H	OH	Cl
1b	O	-	H	CO_2K	H
1c	O	-	H	OH	H
1d	HO,OLi	-	H	CO_2Li	H
2a	CH	O	$CH_2C{\equiv}CH$	Cl	H
2b	CH	O	CH_3	Cl	H
2c	CH	O	CH_3	NO_2	H
2d	CH	O	H	Cl	H
2e	N	O	H	Br	H
2f	CH	O	H	NO_2	Cl
2g	CH	O	H or CH_3	NHOH	H, Cl, F
2h	CH	O	$CH_2CH_2NEt_2$	Cl	F
2i	CH	O	CH_3	NO_2	F
2j	CH	O	CH_2POMe_2	Cl	H
2k	CH	S	CH_2CF_3	Cl	F

Anti-Anxiety Agents — The use of benzodiazepines in the treatment of neurotic anxiety has been reviewed.[15] Diazepam (2b) i.v. was beneficial in treating anxiety symptoms associated with vaginismus.[16] D-Oxazepam hemisuccinate (RV1208, 1c) was superior to the racemic form in human anxiolytic studies.[17] Ripazepam[18] (CI-683, 3) at 40—80 mg. and ketazolam[19] (4) at 46.9 mg were clinically effective in treatment of anxiety and bromazepam (2e) reduced gastric acid secretion related in induced anxiety.[20] A clinical study with BU-1014 (5) in anxiety patients uncovered significant side-effects that lasted two weeks.[21]

In animal studies the 4, 5-dihydro analog of diazepam (2b) was of equal activity[2b] and CRC 2015 (1d) was especially effective against agressive behavior in rodents.[2c]



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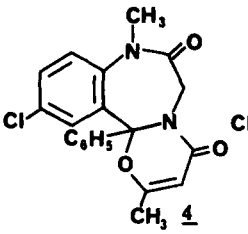

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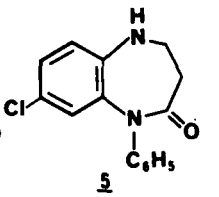

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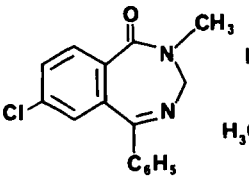

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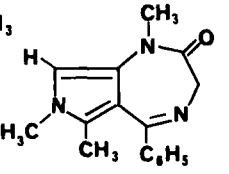

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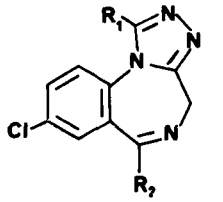


8a R_1=CH_3 R_2=C_6H_4-2-Cl

8b R_1= H R_2=C_6H_5

8c R_1=CH_3 R_2=C_6H_5

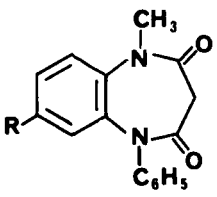
Anticonvulsant Agents — Clonazepam (2f) was clinically useful in the treatment of akinetic and myoclonic seizures,[22] intention myoclonus,[23] tic douloureux[24] and has been recorded as the drug of choice for status epilepticus.[25]

Diazepam (2b) was completely effective against penicillin-induced convulsions in rabbits.[26] The hydroxyamino compounds 2g were generally less active as anticonvulsants than their nitro analogs in mice.[27] Compound 6 was ca. as potent as diazepam (2b) against metrazole included convulsions[28] in mice and the pyrrolobenzodiazepines such as 7 were effective against pentylenetetrazole induced convulsions.[29]

Sedative-Hypnotics — The benzodiazepines have superceded the barbiturates in hypnotic usage[30] with diazepam (2b) the hypnotic of choice for medical ward patients.[31]

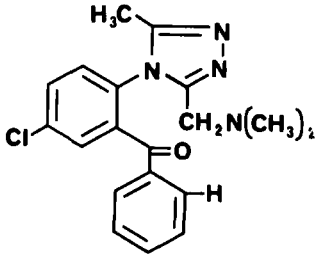
Triazolam (U-33,030; 8a) was an effective hypnotic in insomniacs[32—36] at 0.5-0.6 mg, but showed loss of effectiveness with intermediate term usage and its withdrawal was followed by worsening of sleep.[37] Estazolam[38] (8b) at 3 mg and D-40TA[39] (8c) at 2 mg. provided sound sleep for preoperative patients.

Lorazepam (1a) in 2 or 4 mg doses showed hypnotic activity in insomniacs[40] and good sedation in surgical premedication.[41] Flurazepam (2h) is useful in the long term treatment of insomnia[42] and shows no rebound effect after withdrawal.[43] Flunitrazepam (2i) has hypnotic activity in man at 2.5 mg, but does not induce physiological sleep.[44] Fosazepam (2j) at 60-80 mg decreased sleep onset and awakening in healthy subjects.[45] Quazepam (Sch 16134; 2k) has been entered in the USAN listing as a sedative, hypnotic.[46] Clobazam (9a) at 10—20 mg, but not triflubazam (9b) was useful for limited sleep difficulties in healthy males.[47]



9a R=Cl

9b R=CF_3



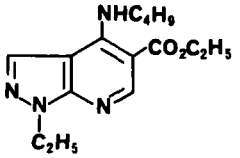
10

A series of aminomethyltriazolyl-, oxotriazolyl- and imidazolybenzophenones were found to be active sedatives, in mice. The potency of triazoles such as 10 which can be regarded as prodrugs of 8a and 8c approached that of diazepam.[48]

Non-Benzodiazepine Compounds

Anti-Anxiety Agents — Cartazolate (SQ 65, 396; 11) at 100 mg daily was less effective than diazepam at 20 mg on various anxiety rating scales[49] and GPA 2640 (12) at 1100—1300 mg daily failed to alleviate anxiety in anxious non-psychotics.[50] Trazodone (13) at 75—150 mg daily was equivalent to 30—60 mg of chlordiazepoxide in relieving symptoms caused by anxiety.[51]

Evaluation in cats suggests Sch 12679 (14) is effective in reducing many forms of agression.[52] DL-254 (15) appears similar to diazepam.[53]



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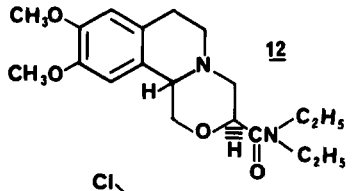

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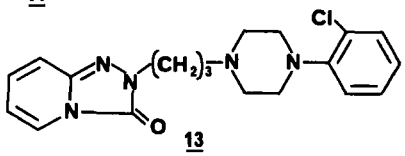

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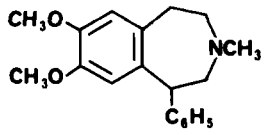

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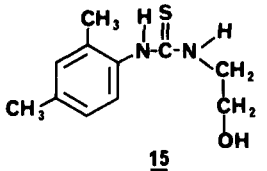

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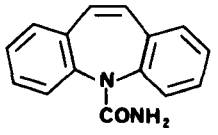
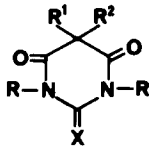


16

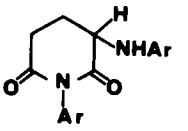
Anticonvulsants — The diagnosis, treatment and most frequent etiologic factors involved in status epilepticus were reported.[54] The effect of antiepileptic drugs on epileptogenesis[55] and the clinical pharmacology of anticonvulsant agents[56] have been reviewed. Anticonvulsant drugs appear to limit the propagation of seizures through the balance of excitatory glutamate pathways and inhibitory GABA and norepinephrin (NE) pathways.[57] A reciprocal relationship between NE levels at receptor sites and audiogenic seizure susceptibility in mice has been observed.[58] A modified maximal metrazole seizure test in mice that can define three main groups of anticonvulsants has been developed.[59]

Carbamazepine (16) at plasma levels of 4—10 μg/ml exerted a remarkable drop in seizure frequency,[60] showed no tolerance after six days at 50 mg/kg daily,[61] was equivalent to diphenylhydantoin[62] and primidone[63] (17) in preventing psychomoter seizures and improved alcohol withdrawal symptoms in male outpatients.[64] Eterobarb (18) is a safe and potent anticonvulsant with a low hypnotic effect.[65]

The pharmacokinetics of vinylbarbital (19) in man[66] as well as 16 and its major metabolite, the 10, 11-epoxide, were studied in children.[67]

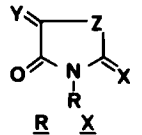


	R	R^1	R^2	X
17	H	C_6H_5	C_2H_5	H_2
18	CH_2OCH_3	C_6H_5	C_2H_5	O
19	H	$CH{=}CH_2$	C_4H_9	O

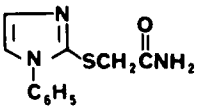


20

The R isomers of glutarimides such as 20 in mice provide a more rapid onset of action and greater anticonvulsant activity and neurotoxicity than the S analogs.[68] Compounds 21—24 were effective in mice against tetrazole induced seizures[69—71] and TMD (22) significantly reduced grand mal convulsions.[72] Pentetrazole convulsions in mice were protected against by 24,[73] TMHT[74] (25), 26,[75] 27,[76] 28 and 29 (R = alkyl; R_1 = aryl).[2d]



	R	X	Y	Z
21	CH_3	O	H,p-$NO_2C_6H_4$	CH_2
22	CH_3	O	CH_3,CH_3	O
23	C_6H_5	S	=CHAr	NH



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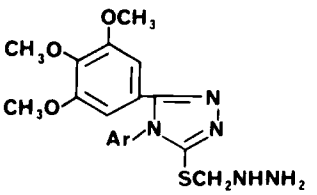

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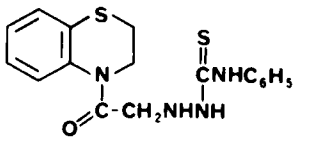

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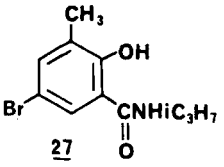

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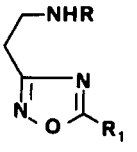

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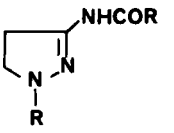
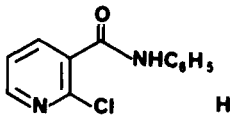


29

Compound 30 had a better therapeutic index than phenobarbital (PB) but appeared less active[77] and 31 was comparable to PB against electrochock, but less effective against pentetrazole convulsions.[78]

Aminoethers such as 32 protected against maximal electroshock seizures in rats.[79]



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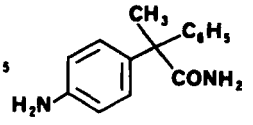

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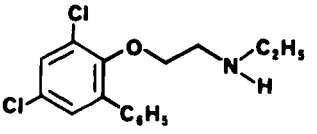
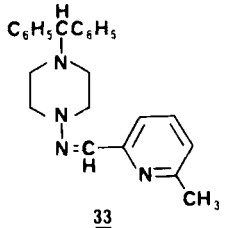


32

Several piperazinoimines with anticonvulsant activity such as 33[80] in light sensitive baboons, 34[81] and 35[82] against tetrazoles, and 36[83] against electroshock, were reported. Structurally related imines such as 37 exhibited inhibition of pentylenetetrazole induced convulsions.[84]



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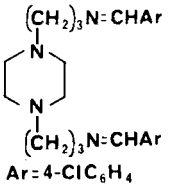

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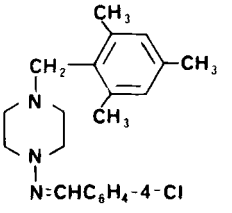

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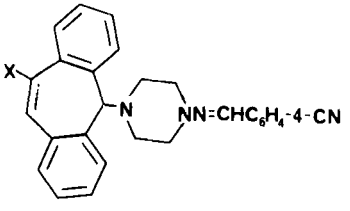
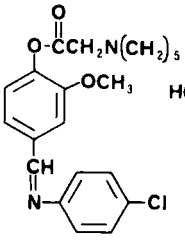


36

X=CN,Br



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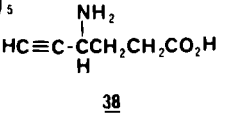

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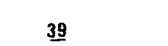
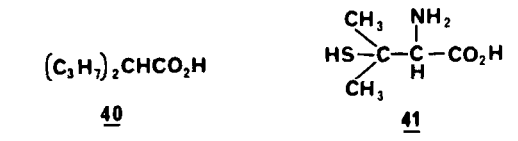


39

GAG (RMI 71675; 38), an irreversible GABA transaminase inhibitor, protected mice against audiogenic seizures, thiosemicabazide (TSC) and electroshock.[85] The anticonvulsant activity of aminoxyacetic acid (39) is manifested through two mechanisms, one involving GABA metabolism,[86] and against TSC it has been shown to be fast in onset and of short duration.[87]

Dipropylacetic acid (40) protected rats against picrotoxin and pentetrazole.[88] The chelating agent D-penicillamine (41) markedly reduced seizures in P. Papio baboons, a primate with high serum Zn levels.[89]



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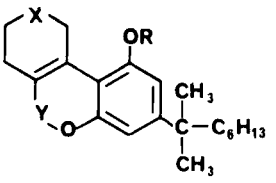
41

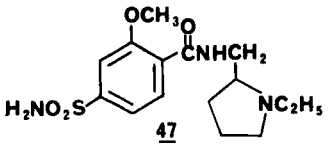


	X	Y	R
42	CHMe	CH_2CO	H
43	CHMe	CMe_2	H
44	CHMe	CMe_2	$CO(CH_2)_3N(CH_2)_6$
45	CO	CMe_2	H
46	$N\text{-}CH_2C{\equiv}CH$	CMe_2	$CO(CH_2)_3N(CH_2)_{5,6}$

Lactone 42 proved equivalent to $\triangle^9$-THC (43) against electroshock in rats.[90] The anticonvulsant activity of A42574 (44) in photosensitive baboons was poor in comparison to its activity in rodents.[91]

Sedative-Hypnotics — The clinical aspects of sleep and psychotropic drugs were reviewed and standardized methodology for measurement was recommended.[92] A model study for obtaining a detailed clinical profile of a hypnotic was proposed.[43] Studies have appeared on the effect of anxiolytic drugs on objective and subjective sleep parameters in healthy normal volunteers,[93] the behavioral performance of habitual long sleepers after an alteration in their sleep schedules,[94] and the induction of symptoms mimicking those of insomnia by coffee and caffeine[95] in normal subjects. Clinical and experimental arguments are summarized in favor of a possible relationship between sleep and memory and between the capacities for learning and paradoxical sleep.[96] The drug interactions of a number of hypnotics and sedatives in clinical use were reviewed.[97] Physostigmine injected during non-REM sleep induced the REM stage, suggesting the role of a cholinergic mechanism in the induction of REM sleep.[98]

The neuroleptic sulpiride (47) at 200—400 mg. i.m decreased time to sleep and awakening, and increased all sleep phases with no significant change in distribution.[99] Diphenylhydramine (48) was a safe and effective sleep aid for pediatric patients at 1 mg/kg.[100] Single doses of 1—2.5 mg. of nabilone (45) induced sedative effects with no appreciable effect on heart rate, lending support to the hypothesis that one can separate out the desirable effects of the cannabinoid class of compounds. L-Tryptophan (49) at 1—5 g orally decreased sleep latency in mild insomniacs and latency and wakenings in normal volunteers.[102] Preoperative sedation with oxypertine (50) was achieved at 20 mg oral dosage.[103] A three week sleep study with mesoridazine (51) gave encouraging results[104] and a new glutarimide, biglumide (52) has entered clinical trials.[105]



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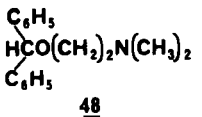

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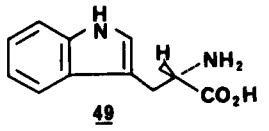

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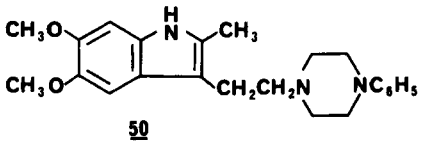

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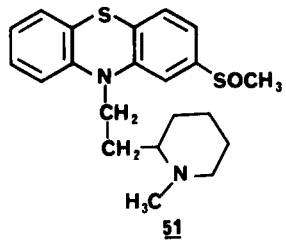
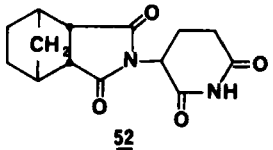


51

Heterocannabinoids 46 have shown sedative-hypnotic and analgesic activity in cats and rodents.[106,107] Compound 53 showed sedation and anticonvulsant activity in mice.[108] Kessoglycol monoacetate (54) was reported to have a sedative-hypnotic profile in animals.[109]



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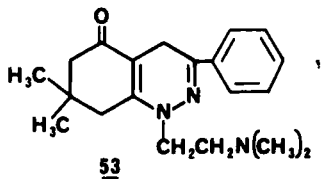

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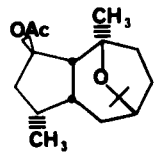

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Chapter 3. Analgesics, Antagonists, the Opiate Receptor and Endogenous Opioids

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The term "opiate receptor" is used to designate areas of the brain having a specific affinity for opiate agonists and antagonists.[1-17] Endogenous peptides called enkephalins and endorphins can react with the opiate receptor[18,19] to produce opiate effects; the effects of these peptides are antagonized by naloxone. In elaboration of the opiate receptor concept, Feinberg et al.[20] have reported a potential model explaining the structure-activity relationships of opiate agonists and antagonists.

The finding that synthetic analgesics like the morphinans and the benzomorphans are capable of interacting in a quite specific way with receptors in the central nervous system, depending on their relative agonist or antagonist properties, suggests that further studies with such synthetic analgesics may provide as useful a tool in unraveling the mysteries of the opiate receptor as the current approaches involving peptide research.[23]

The past year has seen the isolation from mammals of a number of opioid substances in addition to the previously discovered enkephalins and endorphins.[24] Among the substances reported are MLC (morphine-like compound, Levy et al.[25,26]), anodynin (Pert et al.[27]), MLF (morphine-like factor, Pasternak et al.[28]), and pituitary opioid peptide (POP, Goldstein[29]). All of these are peptides with the exception of MLC, which appears to be morphine-like based on the fact that it reacts with antibodies generated against morphine; however, the effects of MLC are not antagonized by naloxone. A number of known peptides have been found either to have partial agonist-like properties (i.e., substance P,[30] somatostatin and ACTH[31]) or to act as prohormones (i.e., β-lipotropin[32,33]).

The intensive research now ongoing in the opiate receptor area has led to suggestions that the endogenous opioids are not only involved in analgesia but may also play a role in mental diseases such as schizophrenia and depression. Some recent reviews in the area have been published.[21,22] Guillemin[34] has suggested that brain peptides are linked to schizophrenia since the catatonia produced by these peptides in animals[35] is very reminiscent of schizophrenic catatonia. Naloxone reverses the catatonia and hypothermia produced by brain peptides in animals and in humans. Indeed Gunne[36] has reported that naloxone antagonizes auditory hallucinations in schizophrenic patients. Byck[37] has suggested that the actions of morphine (analgesia, sleep, euphoria, and respiratory depression) are simulated by various transmitter or modulator substances in the brain. Thus, enkephalin is proposed as a neurotransmitter and its binding to opiate receptors determines mood state and influences respiratory and sleep patterns. Lithium may act through modification of the binding of the endogenous morphine-like substances at the opiate receptor. This theory would also predict the blocking of mania and most drug-induced euphorias by naloxone.

Endorphins. The endorphins have been defined as a class of opioid-acting peptides isolated from brain, all of which seem to be fragments of β-lipotropin.[22,39] The endorphins have the following structures:[40]

α-Endorphin (β-lipotropin$^{61-76}$)

H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-OH

β-Endorphin (β-lipotropin$^{61-91}$)

α-Endorphin-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
γ-Endorphin(β-lipotropin 61-77)
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-OH
The brain-derived β-lipotropin fragment α-endorphin has analgesic and tranquilizing activity in animals, whereas γ-endorphin, which differs from α-endorphin only in having a leucine residue added to the C-terminal end of α-endorphin, induces violent behavior when injected into animals. Finally, the larger fragment, β-endorphin, also produces analgesia,[41] profound sedation and catalepsy[42] in animals. β-Endorphin has 48-400 times the analgesic or catalepsy producing activity of morphine when injected into the brain,[43, 44] three times the activity of morphine when injected intravenously, and its effects are blocked by naloxone. The interest in β-lipotropin is further stimulated by the fact that its 61 to 65 sequence is identical to that of Met-enkephalin.[45] However, no precursor has yet been found to Leu-enkephalin.

A totally synthetic β-endorphin[46] was shown to be identical with the natural material both chemically and biologically,using the membrane fraction from rat brain homogenate to assay for receptor binding and the rat tail flick test for analgesic activity. Synthetic α-endorphin and γ-endorphin have been synthesized[47] and these have been shown to be identical with the natural materials. It has been reported that β-endorphin not only is a potent analgesic agent but also is capable of inducing physical dependence of the opiate type.[13,48-50,52,53] When infused for 70 hrs into the periacqueductal gray-fourth ventricle spaces of the rat brain, methionine-enkephalin and β-endorphin produce a typical morphine-like withdrawal syndrome when challenged with naloxone.[29,55] β-Endorphin can also produce analgesia when injected into mice intravenously.[56] β-Endorphin is found in considerable quantity in the pituitary.[22,58] The β-endorphin found in the sheep and camel pituitary is identical with that found in man, except the latter has tyrosine replacing histidine at the 27th position, and glutamic acid replacing glutamine at the C-terminal end (position 31). β-Endorphin has a high affinity for brain opiate receptors as measured by competition with tritiated naloxone and dihydromorphine for binding to a washed membrane preparation.[59] The removal of residues 30 and 31 (Gly-Glu) had little effect and 28 and 29 (Lys-Lys) removal had a profound (20-fold reduction) effect on the binding properties of β-endorphin. Other pituitary and brain factors differ from β-endorphin in their greater potency,[60] destruction by trypsin and insensitivity to cyanogen bromide. Thus, the entire endorphin puzzle is a long way from being solved.

It has been speculated[60] that classical hormonal feedback mechanisms might act to suppress endogenous opioid synthesis when the receptors are occupied by an endogenous opiate-like morphine.[61] Enkephalin levels in the brain of morphine-tolerant rats are increased,[61] and there is cross-tolerance between morphine and Met-enkephalin.[62]

Although the block of both opiate and endorphin effects by naloxone shows a similarity between them, there are still some observations relating to the opiate receptor that cannot be put in any context. Thus, it is difficult to explain why naloxone antagonizes the analgesia produced by focal electrical stimulation of the brain[63] or by acupuncture,[64] yet does not antagonize analgesia in man produced by hypnotic suggestion. Nor does naloxone show any significant disruptive effects on shock escape threshhold

or temperature control under cold stress in rats. On the other hand, naloxone does block food-seeking and water-seeking behavior in hungry or thirsty rats. The fact that biogenic amine modifiers do not affect morphine analgesia and naloxone antagonism by a similar pattern also indicates that our concepts of the opiate receptor interactions are incomplete.[65,66] All three endorphin peptides and Met-enkephalin produce in drug-free animals the "wet-dog" shaking seen in opiate withdrawal, and these responses are antagonized by naloxone.[43] It has been suggested that β-endorphin and Met-enkephalin play a role in growth hormone secretion[67] and in cyclic AMP.[68]

Stimulation of the central gray region in humans has produced marked analgesia which is antagonized by naloxone. This phenomenon has led to speculation that there is ongoing release in the brain of morphine-like compounds which is partially responsible for elevating pain thresholds. However, perception of experimentally induced pain in human subjects is not altered by naloxone administration.[69] Perhaps an interaction with acetyl choline, with nor-adrenalin release,[51] with the adenylcyclase system[57,70,86] or neural firing[38,71] is leading to these complex, unexplained phenomena.[72,73] Finally, an electrically induced release of endogenous opiate receptor ligand has been reported, including reversal by naloxone.[74]

Enkephalins. A subset of the endorphins are called enkephalins.[75-80,82-88] Although the various enkephalins have agonist activity on appropriate administration,[62,90] they are less potent than the endorphins and are thought to be enzymatic degradation products of endorphins, or perhaps even artifacts produced in the isolation technique. Endorphins may be produced by enzymatic cleavage of β-lipotropin, and there is speculation[91] that a lack of this enzyme "may be an etiological factor in those psychopathological states for which the exogenous neuroleptics exert an ameliorative influence."

Met-enkephalin[92] (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) have lower *in vivo* potency of any of the endorphins.[94] The enkephalins are quite short-acting *in vivo* because they are degraded by peptidases in blood and brain.[95] Synthetic enkephalins have been prepared which are identical to the natural materials.[96,97] Goldstein[98] has prepared a heptapeptide H-Tyr-Gly-Gly-Gly-Lys-Met-Gly-OH based on its spatial resemblance to morphine. However, its potency was very low. Steric analyses of the enkephalins have been published[54,89,99-101,159] and it is concluded that there is no single conformation in solution. A series of enkephalin analogs were synthesized and their affinities for opiate receptors compared. Met-enkephalin has 1/3rd the potency of morphine, but three times the potency of Leu-enkephalin.[100,104] A synthetic enkephalin[81,102] [D-Ala^2]-Met-enkephalinamide (DALA) binds to opiate receptors almost as strongly as Met-enkephalin. Since DALA is not susceptible to degradation by brain enzymes doses of 5-10 mcg cause profound, long-lasting, morphine-like analgesia when injected into rat brain. Resistance to enzyme degradation[103] is retained if D-Alanine is replaced by other D-amino acids, by L-proline or by sarcosine.

Replacement of methionine by norleucine gave an analog with about 50% of the potency of the parent, but replacement of glycine or -OH led to marked loss of activity. Substitution of tyrosine by phenylalanine, blocking of the hydroxyl, or removal of the amino group practically abolished activity. Replacement of the Phe- by Tyr- also practically abolished acti-

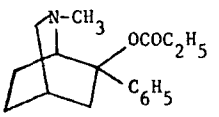
vity.[104,105] The peptides larger than Met-enkephalin are not appreciably more active when measured on the isolated guinea pig ileum. However, the much greater activity of the endorphins compared with Met-enkephalin in vivo is probably due to the protective effect of the longer chain. Additional receptor binding sites may also be present within residues 80-91 of LPH (61-91).[105]

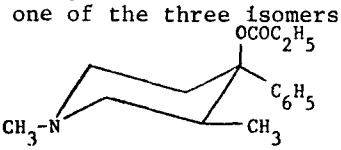
Anodynin. Of major importance to the current of research on opioid peptides in brain is the finding of an endogenous opiate analgesic, anodynin, in human plasma[106] and in rat brain. Anodynin differs from the enkephalins in its longer duration of action, its lack of susceptibility to enzymatic splitting, its thin layer mobility, its behavioral effects and its apparant ability to cross the blood brain barrier. It has in common with enkephalin its reversal by naloxone. The pituitary origin of anodynin is suggested by the greatly reduced levels found in rat blood after hypophysectomy. It is unlike enkephalin in this respect.

Miscellaneous. A long-acting, polymer-bound form of naloxone[107] has been prepared. Naloxone, attached to a hydrazine-substituted polysaccharide by a hydrazone bond, antagonizes morphine analgesia more than 25 times longer than free naloxone.

Using the spinal dog as a research tool, Martin[108] has postulated at least three different agonist receptor sites, each associated with a particular agonist syndrome. According to Martin's classification:
1) (μ) agonist receptor - morphine-like effects like euphoria and sedation
2) (κ) agonist receptor - nalorphine or cyclazocine-like agonist activity
3) (σ) agonist receptor - associated with psychotomimetic effects. Cyclazocine and nalorphine are mixed (κ) and (σ) agonist, ethyl keto-cyclazocine is a pure (κ) agonist, and morphine is a pure (μ) agonist.

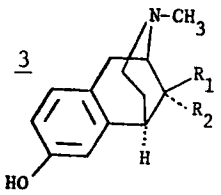
Prodines and Related Structures. Among analogs of the prodine-type, the most potent compound (1) was found to be half as potent as an analgesic,[109] but twice as toxic as alphaprodine (2). The ED_{50} (tail flick) for 1 and 2 are 3.1 and 1.4 mg/kg, respectively. Only one of the three isomers of 4-

1 

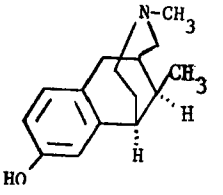
2 

acetoxy-1,2,6-trimethyl-4-phenylpiperidine was an effective analgesic in mice (2.3 x meperidine) as judged by the hot-plate test.[110] Alpha(+)allylprodine was 40 times more potent than morphine, 260 times more potent than (-)allylprodine and 460 times more potent than its β diastereomer.[111] The relative brain levels of meperidine and three N-alkyl homologues determined at equal analgesic iv doses in mice were found to be closely proportional to their ED_{50} doses in spite of the wide differences in partition coefficients and in rates of metabolic N-dealkylation. It was postulated, therefore, that the observed ED_{50} potencies provide a fair comparison of the relative receptor affinities of the four homologues.[112]

Benzomorphans. The 9βanalog (3a) of the isomeric 2,9-dimethyl-2'-hydroxy-6,7-benzomorphans was 4 times as potent (ED_{50}=1.1 mg/kg) as the 9α analog (3b) by the hot plate test.[113]

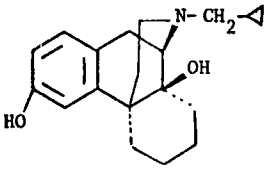


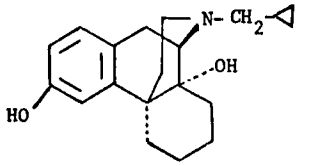
3a $R_1 = CH_3$, $R_2 = H$
3b $R_1 = H$, $R_2 = CH_3$
3c $R_1 = C_3H_7$, $R_2 = H$
3d $R_1 = H$, $R_2 = C_3H_7$



The 9β-propyl levo isomer (3c) was considerably more potent subcutaneously than morphine, while the 9α-propyl levo isomer (3d) was equipotent with morphine as an analgesic.[114] None of the optical isomers suppressed withdrawal signs in monkeys; the 9β-propyl levo isomer exacerbated the withdrawal syndrome, indicating that it possesses some narcotic antagonist activity.[114] The most active compound (4) of six homobenzomorphans was as potent subcutaneously as morphine (measured by pressure stimuli on mouse tail).[115,116] Simplified procedures for the synthesis of the 6,7-benzomorphan series have been described.[117,118] Several 11-hydroxy and 11-alkoxy-2,6-methano-3-benzazocines display as potent analgesic and narcotic antagonist activity as cyclazocine.[119]

Morphinans. Only one of the two enantiomeric quaternary iodides, N-methyl-levorphanol exhibited specific opiate effects while the other enantiomer, N-methyl-dextrorphan was, as expected, inactive.[120] In a series of 14-hydroxymorphinans, the epimeric isomorphinan (6) was a more potent antagonist than oxilorphan (5)(BC-2605).[121] A review on the pharmacology of butorphanol (cyclobutyl analog of 5) has been published[122] and clinical investigation of both the oral and parenteral forms is continuing.[123-128]

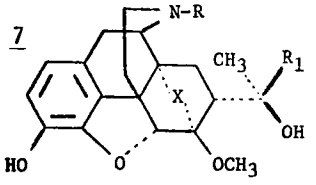




Morphines. It has been shown that removal of the N-methyl group leads to a considerable loss in agonist activity as measured by the guinea-pig ileum method with two exceptions; morphine and normorphine, as well as codeine and norcodeine, are equiactive in this test.[129]

Morphine was found to be 6 times more potent as an analgesic and approximately twice as lethal as morphine-6-hemisuccinate after ip administration.[130]

Examination of the role of the gut in the metabolism of three phenolic analgesics, dihydromorphine, etorphine (7a), and buprenorphine (7b), which possess widely differing partition coefficients (κ)(heptane/phosphate buffer pH 7.4), revealed that a major pathway (conjugation) exists in the gastrointestinal tract for the protection of the organism from the potential toxic effects of phenolic substances and that a determining factor in



(7a) R=CH_3, R_1=nPr, X=-CH=CH-, κ= 0.15)

(7b) R=CH_2  , R_1=tBu, X=-CH_2-CH_2-, κ=1.78)

(7c) R=$CH_2CH=CH_2$, R_1=n-Bu, X=-CH_2CH_2-

the efficiency of this pathway is the lipophilicity of the substance.[131]

The results of this study suggest that the role of the liver in the deactivation of potent agonists after oral administration has sometimes been overemphasized, for it has been shown that this organ is not essential for the "first pass" metabolism of the lipophilic compounds buprenorphine and etorphine in the rat. Dihydromorphine (K=5.0) appears not to be readily conjugated by rat intestine and the liver must be regarded as the prime site of metabolism of this relatively polar compound.[131]

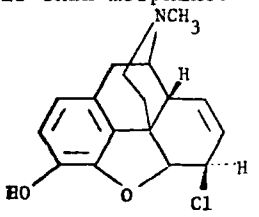
The analgesic effects of R+S 218-M (alletorphine)(7c) administered in a dose of 0.56 mg/70 kg was as effective as 10.5 mg/70 kg of morphine sulfate. No evidence was found to support the claim that alletorphine (7c) causes less respiratory depression when compared with morphine sulfate.[136]

The relative agonistic and antagonistic potencies of naloxone, naltrexone and their 6-methylene derivatives were evaluated in the guinea-pig ileum. All compounds were competitive antagonists of azidomorphine, the morphine-like agonist used in the experiments. The 6-methylene substitution caused approximately 50 and 100% increases of the antagonistic activity of naloxone and naltrexone, respectively. All compounds have little or no agonistic activity.[132]

In man, azidomorphine constricted pupils, produced morphine-like subjective effects and euphoria, and suppressed the morphine abstinence syndrome. It was concluded that in man azidomorphine is a typical morphine-like drug.[133]

The α(8) and β-halomorphides (9) possessing a C-6 axial and C-8 equatorial halide, respectively, were found to be more potent analgesics and also more toxic than morphine.[134]

8



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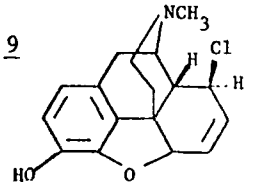

The disposition, metabolism and effects after acute and chronic dosing of naltrexone were investigated in 4 subjects, showing that narcotic antagonism was related to plasma levels of naltrexone.[135]

Aminotetralins. In continuing studies on new bridged aminotetralin analgesics, the best activity among esters, N-oxides, and compounds containing a butenyl or a benzo-bridge was seen for (10), which was 2.5 times as potent as morphine.[137] Resolution of the more potent analgesics of the bridged aminotetralin type produce WY-16225 (the (-) enantiomer of (11)) which was 8 to 15 times as potent as morphine when administered parenterally. Desocine (11) was judged to be a morphine antagonist slightly less potent but longer acting than nalorphine based on physical dependence studies carried out in morphine-dependent Rhesus monkeys.[138] This compound

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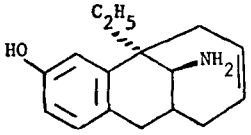
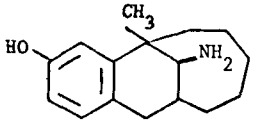


11
(Dezocine)



is currently undergoing clinical trials.

In continuing studies on aminotetralin derivatives the best compound, (12), was found to possess slightly more than half of the oral analgesic potency of morphine as measured by the acetic acid writhing test.[139]

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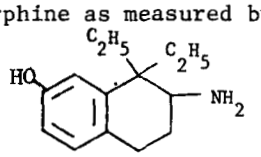

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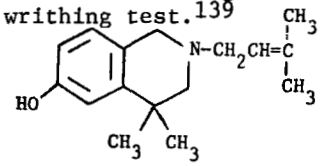
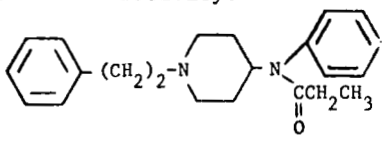


Miscellaneous. In the seventh publication of ongoing studies on tetrahydro-4,4-di-methylisoquinoline type analgesics, the analgesic activity of (13) was found to be slightly more potent than that of codeine.[140]

In an attempt to enhance the analgesic activity of fentanyl (14), cyclization of the acyl group with C-2 of the aromatic ring was carried out. Compound (15) did not show any analgesic activity. It did show, however, strong antihistaminic activity.[141]

14



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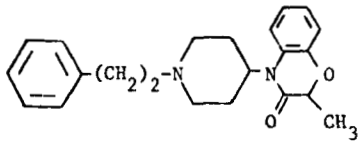

The best analgesic activity of a series of 10,11-dihydrodibenzo[b,f] thiepin derivatives was displayed by (16), without listing a comparative value of a reference standard.[142] The compound also displayed other pharmacological activities.

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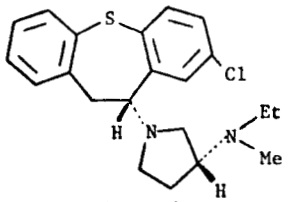

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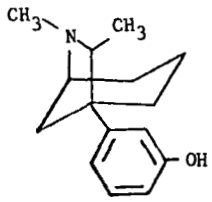


In a series of azabicycloalkanes, (17) and its dextro isomer exhibited analgesic activity comparable to meperidine and morphine, respectively.[143] The two compounds also exhibited narcotic antagonist activity. (17) Produced slight physical dependence capacity in the Rhesus monkey.

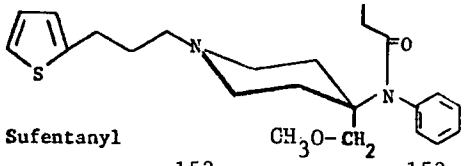
The relative binding affinities of the optical isomers of methadone, α-methadol, α-acetylmethadol and their N-demethylated derivatives to the opiate receptors of rat brain confirmed the agonistic nature of this series of drugs.[144]

In azabicyclo[3,3,1]non-6-ene series some compounds showed analgetic activity in the acetic acid induced writhing test.[145]

Sufentanyl (R-30,730)(18) was the most potent compound in a novel series of 4-substituted fentanyl derivatives. Sufentanil, which is 4500 times more potent than morphine, has a rapid onset but relatively short duration of action and its margin of safety is reported to be unusually high (LD_{50}/ED_{50}>25000).[146]

Reports on research with phenylmorphans,[147] meptazinol (a homopiperidine),[148,149] isoxazoloquinazolinones,[128] biphenyl Mannich bases,[151] 5-

18



Sufentanyl

methyl methadone diastereoisomers,[152] 3-isothujone,[153] α-promedol enantiomers,[154] benzoisoquinolines,[155] diphenylethylpiperazines,[156] and piritramide[157] have appeared.

Cannabinoid Compounds. The continuing studies on heterocyclic and carbocyclic analogs of cannabinoids yielded compounds which show significant analgesic activity. Many of these compounds also exhibit sedative, antihypertensive or anticonvulsant activity.[82,93,150,158]

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Chapter 4. Memory and Learning - Animal Models

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Introduction. This review focuses primarily on the effects on memory of hormones and central norepinephrine. There has been considerable interest lately in the roles of hormonal and catecholaminergic systems in the storage of information provided by a new learning experience. Many treatments, including hormones, drugs which alter neurotransmitter functions, electroshock, and localized direct electrical stimulation of the brain, can modify retention.[1,2,3,4,5,6] In these studies, a treatment is typically administered at some relatively short time after training (e.g., 0 - 6 hrs) and animals are tested for retention at a later time (usually more than 24 hr after training). The general finding is that, if a treatment affects retention, the effectiveness of the treatment decreases as the time after training is increased. Depending on the treatment and on the specific experimental procedures, the effect, on retention, may be either impairment (retrograde amnesia) or enhancement of memory. The time after training during which retention loses its susceptibility to modification by a particular treatment is termed the retrograde amnesia (or enhancement) gradient. In the past, a great deal of theoretical interest in this research focused on the time-dependent nature of these effects.[7,8] It was thought that the time-course of retrograde amnesia enhancement gradients reflected a biological constraint on the time necessary to complete memory storage, i.e., the time required to form long-term memory. A time-constant of this sort would have critical implications in terms of the neurobiological mechanisms which might be assigned the function of memory storage. Obviously, different mechanisms of storage would be involved for a process which required only a few seconds as compared to one which required several hours to reach completion.

It is therefore somewhat unfortunate that this information is not likely to be forthcoming from studies of retrograde amnesia or enhancement. Rather, it now appears that for any learning situation, the time after training during which memory may be modified is determined to a large extent by the severity (i.e., drug dose, electroshock intensity, number of treatments) of the treatment. In some studies using animals, the gradient may vary from a few seconds to hours or days.[9,10,11] In humans, there are indications that the retrograde amnesia gradient may be as long as several years.[12] Because the gradients are not "fixed" (biologically) for a particular learning situation, it is preferable to think of the time-dependent effects on memory as reflecting decreasing susceptibility to modification with time after training.[13] An extension of this view is that the treatments are modulators of memory processing, altering the activity of systems which are responsible for promoting the storage of specific information.[14]

In addition to the theoretical implications of this research, there is also a practical advantage to the use of the general experimental procedures described above. Studies which examine the effects of a treatment on memory processing are perhaps best suited to do so if the treatment is administered shortly after training. This design assures that all animals

are untreated at the time of training. Furthermore, with the addition of delayed treatments which do not alter retention performance, one can conclude that any effects on retention performance are the result of retroactive modification of memory storage processing and are not the consequence of a direct proactive influence of the treatment at the time of memory. Thus, the general experimental design excludes possible alterations in perception or motor capabilities at the time of training or testing as a viable interpretation of the changes in retention.[3]

Hormonal manipulation. The general interest in the study of hormonal manipulation effects on memory was fostered by the recognition that these systems are responsive to many if not all training situations[15, 16, 17, 18] and therefore have the potential of participating in both physiological adaptation to environmental demands as well as the behavioral adaptation to these demands.[19, 20, 21] Furthermore, the hormonal role in adaptive behaviors may include effects on learning,[22] on memory storage,[23] or on maintenance of learned responses.[24, 25]

ACTH, ACTH analogs, and vasopressin. Much of the earlier literature in this area has been reviewed previously.[21, 26, 27, 28, 29] These studies clearly indicate that ACTH injections may delay extinction of learned responses (i.e., a decrease in a previously learned response in the absence of reward or punishment), although it is not clear whether this result is due to potentiation of previously learned responses or to impaired acquisition of the extinction training. These behavioral effects have been observed during extinction of avoidance responses[21, 30] as well as extinction of rewarded behavior.[31, 32, 33] In one study for example,[31] rats were trained to approach food in an alleyway. Following training, animals received daily injections of porcine $ACTH_{1-39}$, synthetic $ACTH_{1-24}$ (both of which have adrenocortical activity), $ACTH_{4-10}$ (which does not have adrenocortical stimulating activity) or corticosterone, prior to extinction training. ACTH and its analogs retarded extinction, while corticosterone accelerated extinction, as compared to placebo-treated controls. In addition, ACTH injections may alter acquisition rates of avoidance training.[34, 35]

A series of experiments examined the role of pituitary-adrenal hormones in acquisition and extinction of conditioned taste aversion learning.[36, 37, 38] In this behavioral situation, an animal is allowed to ingest a solution with a specific (typically novel) taste. At some time within the next several hours, the animal receives an injection of lithium chloride or other agent. When tested at a subsequent time, the animal will show an aversion for the solution. The findings of this set of experiments revealed that plasma corticosterone levels in male rats were elevated for up to 4 hrs after an appropriate injection of lithium chloride, indicating activation of the pituitary-adrenal system under these training conditions. Pretreatment with dexamethosone, a steroid which inhibits ACTH secretion, blocked the corticosterone response to training and, further, diminished the extent of aversion to the test solution as measured at later time. Under comparable training conditions, ACTH administered prior to the lithium chloride injections had no effect on acquisition, although potentially enhanced acquisi-

tion may have been masked by the fact that control animals ingested very little of the test solution. However, ACTH injections administered prior to extinction sessions did delay the recovery from taste aversion.

Another set of experiments examined the possible modulatory effects of ACTH on memory processing. The possibility, discussed in the introduction, that some treatments may modulate memory storage predicts that there may be endogenous memory modulatory systems which are activated by those post-trial treatments which act to impair or to enhance memory; one such set of systems might include hormonal responses to training. The rationale guiding these studies is as follows: If, for example, an animal is trained in a one-trial inhibitory (passive) avoidance task, retention performance will vary with the footshock intensity. In addition, the extent of the hormonal response to training with different shock levels will vary. The question posed here was: To what extent is better retention performance after training with high footshock a function of the hormonal response to training? The approach used to address this question was to train animals with a weak footshock and to follow the training with subcutaneous injections of ACTH. The hypothesis guiding this study was that posttrial ACTH injections should enhance retention performance by adding to the normal hormonal consequences of training, thereby mimicking the consequences of more intense training and increasing retention performance. The findings of such experiments[39, 40] are generally consistent with this view. Animals which received immediate posttrial injections of ACTH had enhanced retention performance. Delayed injections were ineffective. Somewhat surprisingly, high ACTH doses produced amnesia. The doses used proved to be critical; the dose-response curve was an inverted-U function. Furthermore, the optimal doses for enhancing retention varied in a meaningful manner with the stress (shock level) used in training. A single dose of ACTH enhanced retention of weak footshock training and impaired retention of strong footshock training. Such data support the view that there is an inverted-U relationship between posttrial circulating ACTH levels (endogenous ACTH + injected ACTH) and memory processing. The inverted-U dose-response curve is characteristic of other treatments which enhance memory.[41, 42, 43, 44] Also, the interaction of the optimally enhancing dose of these drugs and other treatments with training-related stress seems to have some considerable generality.[44, 45, 46, 47] Such findings may be related to the inverted-U relationship between stress and performance which has been noted before,[48, 49, 50] and may suggest that the biological dimension underlying the inverted-U may be hormonally mediated. More generally, these findings support the view that hormonal responses to training may modulate memory storage processing.

The mechanism by which ACTH acts on learning and memory is not clear. The hormonal action does not, however, appear to depend on adrenocortical activity. Many studies have demonstrated that melanonyte stimulating hormone, which shares a behaviorally active peptide sequence with ACTH, as well as several N-terminal ACTH analogs (e.g., $ACTH_{4-10}$ [Met-Glu-His-Phe-Arg-Try-Gly]) which have no corticotrophic activity, have effects on learning, memory, and maintenance of learned performance which are quite comparable to those of ACTH itself.[47, 51, 52] Recent studies have examined the

behavioral and biological effects of these agents. The agents do not appear to affect spontaneous motor behavior[51,54] but there is some evidence that the agents may enhance attention in rats[55,56] and in man.[57,58] $ACTH_{4-10}$ alters the frequency and excitability of hippocampal theta waves,[59,60] and alters visual evoked responses in rats[53] when injected subcutaneously. Some ACTH analogs (e.g., $ACTH_{4-9}$ containing amino acid substitutions [Met(O)-His-Phe-dLys-Phe]) are effective behaviorally at far lower doses than those needed for ACTH itself.[61] The peptide doses which are effective appear to be related to an increase in in vitro half-life.[61,62] ACTH and some analogs appear to increase brain RNA and protein metabolism,[63,64,65] glucose metabolism[66] and noradrenaline turnover.[67,68] Thus, it is clear that the ACTH analogs which enhance learning and memory have the capacity to act on the brain, possibly at the level of the posterior thalamic nucleus parafascicularis,[69,70] but the multiple biological effects complicate the issue of which mechanism of action is related to the behavioral effects obtained with these agents.

Other examinations of the relation of ACTH and memory studied the effect of ACTH (or its analogs) on animals rendered amnestic by carbon dioxide exposure (to respiratory arrest) or electroconvulsive shock. If amnestic animals receive an injection of $ACTH_{4-10}$ 1 hour prior to retention testing, the amnesia is significantly attentuated.[71,72]

Vasopressin. Vasopressin (a posterior pituitary peptide [H-Cys-Tyr-Phe-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Lys-Gly-NH_2]) has many behavioral effects comparable to those observed with ACTH. A posttrial injection of vasopressin enhances retention of inhibitory avoidance training[73] and impairs extinction of avoidance training.[74,75] In general, the major difference between the effects of ACTH and vasopressin on learning and memory is that ACTH must be administered throughout training or extinction trials to enhance acquisition or retard the development of extinction. However, a single injection of vasopressin prior to training or extinction trials has comparable effects. Thus, vasopressin effects on learning and memory seem to be of much longer duration than those of ACTH.[75]

It was recently reported that a posttrial injection of vasopressin may enhance later retention of inhibitory (passive)avoidance training in rats with hereditary hypothalamic diabetes insipidus that are not able to synthesize vasopressin.[76,77,78] This effect is time-dependent; delayed injections do not affect later retention performance. Furthermore, in normal Wistar rats, intraventricular injections of antibodies to arginine-8-vasopressin 30 min prior to or immediately after the training trial produce amnesia in dose-dependent manner.[78]

Catecholamines. Peripheral catecholamines have generally received less attention than pituitary hormones in terms of involvement in learning and memory. However, the effects on memory of posttrial epinephrine and norepinephrine appear comparable to those obtained with ACTH or vasopressin.[39,79] Epinephrine injections administered shortly after inhibitory

avoidance training enhance later retention performance and delayed injections (e.g., by 2 hrs after training) are ineffective.[79] Furthermore, the dose-response curve for the effects of epinephrine on memory is an inverted-U; high doses produce retrograde amnesia. The memory enhancing effects of epinephrine are blocked by pretrial injections of the beta-adrenergic blocking agent, propanolol, but are unaffected by pretrial injections of the alpha-adrenergic blocking agent, phenoxybenzamine. Conversely, the amnesia produced by epinephrine is blocked by phenoxybenzamine but not propanolol. Thus, the rising and falling portions of the inverted-U dose-response curve may be mediated by different pharmacological mechanisms of epinephrine.[80]

The role of central norepinephrine and catecholamines, particularly in memory, has been investigated in more detail.[81, 82, 83] Many of the recent studies testing the view that central epinephrine is important to memory processing have used synthesis inhibitors such as diethyldithiocarbamate (DDC), a dopamine beta-hydroxylase inhibitor. This drug is a potent amnestic agent which produces retrograde amnesia in rats, for example, even if injected 24 hr after training,[83] at an intraperitoneal dose (900 mg/kg) which reduces whole brain norepinephrine concentrations to approximately 15% of control levels. The drug produces amnesia in rats and mice for both inhibitory avoidance and visual discrimination tasks.[83, 84, 85, 86, 87, 88] Furthermore, the amnestic effects of a pretraining DDC injection can be attenuated with immediate posttrial intraventricular injections of norepinephrine.[85] It should be noted that although these collective results are consistent with the view that the effects of DDC on memory are a consequence of the decrease in norepinephrine concentrations, there are other effects of the drug which may be important as well.[83] For example, DDC produces a short-term increase in whole brain dopamine and, because DDC chelates zinc, the drug injection results in bleaching of a sulfide silver stain in the hippocampal mossy fibers.[84] It is therefore possible that the effects on memory of this drug may be related not only to the interference with norepinephrine synthesis but also to interference with the activity of other neurobiological systems.

The evidence that treatments which acutely interfere with central noradrenergic systems also impair memory processing seems well founded. However, chronic depletion of central norepinephrine (e.g., with 6-hydroxydopamine or locus coeruleus lesion) does not reliably result in impaired acquisition or retention.[85, 86] Therefore, although telecephalic norepinephrine is not necessary for learning and retention, it appears that, if available, the central noradrenergic systems may modulate memory processing.

Conclusions. There is now considerable evidence that peptide hormones and catecholamines have the potential to act on acquisition, on the maintenance of learned responses, and on memory processing. The neurobiological mechanisms by which these systems act is less clear. The problem is not that central effects of the hormones and drugs are unknown but, rather, that too many effects are recognized. Therefore, it is difficult to assess which of this large set of events mediates the hormonal and aminergic

modulation of learning and memory. However, we can presumably look to the future for studies which pursue these biological mechanisms, perhaps using the strategy of selectively manipulating a particular consequence of a hormonal or catecholaminergic activity independently to assess which neurobiological effects of these systems are related to learning and memory.

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Section II - Pharmacodynamic Agents

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Chapter 5. Antiarrhythmic and Antianginal Agents

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Antiarrhythmic Agents

It has long been believed that cardiac arrhythmias result from changes in the conducting properties and/or automaticity of the myocardium. Normally, each cardiac impulse arises in the sinoatrial node in the right atrium and then is rapidly transmitted throughout the atria and via the atrioventricular node, His bundle, bundle branches and specialized conduction fibers (Purkinje fibers) to all regions of the ventricles to assure coordinated activation and contraction.

The concentration of potassium is considerably higher and that of sodium lower within cardiac cells than in the extracellular fluid. External membranes of cardiac cells are selectively permeable to ions and contain biochemical pumping mechanisms which can transport ions against electrical and concentration gradients. These factors lead to an electrical potential difference across the membrane with the inside negative with respect to the outside. An impulse can arise in the sinoatrial node or other "automatic" or "pacemaker" cells as a result of slow spontaneous depolarization. Usually, when the level of depolarization reaches a critical "threshold" level, self perpetuating changes occur which tend to depolarize the cell completely and even reverse the potential to inside positive. The juxtaposition of depolarized and polarized regions alters the membrane characteristics of the latter and, in turn, causes their depolarization and results in the development of a propagated impulse. Repolarization occurs after a certain period and the cycle recurs.[1-3]

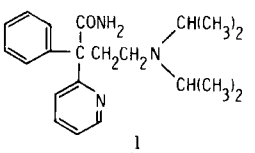
As just outlined, both impulse initiation (automaticity) and conduction result from changes in the permeability of the external membrane of cardiac cells to various ions. Normally, impulse propagation in most cardiac cells results from a rapid but brief increase in sodium permeability which permits an influx of that ion and produces rapid depolarization. As cells become partially depolarized, either as a result of the normal "fast" sodium inward current or for other reasons, other changes in membrane permeability occur leading to a more slowly developing and smaller inward ion flow composed primarily of calcium. In diseased and/or partially depolarized cells this "slow" calcium current may be the only mechanism available for impulse propagation. It may also be responsible for impulse initiation in areas not normally automatic.[1-5]

The electrophysiological characteristics, e.g., excitability,

refractory period, conduction velocity, etc., of even the normal ventricular conduction system are not uniform. Factors which enhance this disparity foster the development of arrhythmias.[1,3,5-7] Ischemia, stretch, catecholamines, potassium, etc. can cause nonuniform or regional suppression of normal conduction mediated by the "fast" response and result in the predominance of the "slow" response with its attendant susceptibility to blockade and automaticity. For example, coronary artery occlusion in dogs results in highly complex arrhythmias.[1,3,5-7] The initial phase occurring within the first few hours is probably due to depressed conduction and reexcitation in the ischemic area. The later phase starting at 12 to 16 hr is probably the result of repetitive impulse initiation in ischemic Purkinje fibers.

Antiarrhythmic drugs have been classified on the basis of their effects on the rate of depolarization, action potential duration, inhibition of the "slow" current, beta-adrenergic blockade, etc.[2] However, it has become apparent that the conditions under which drugs are assessed, i.e., potassium concentration, perfusion with blood in contrast to Tyrode's solution or the use of diseased rather than normal tissue, can affect results greatly.[1,2,8] For example, lidocaine and propranolol markedly depress conduction selectively in ischemic zones but not in normal regions during the early phase following coronary artery occlusion in dogs and thereby suppress arrhythmias.[7]

Clinical reports - Beneficial effects of disopyramide (1) in various human arrhythmias were described in recently published symposia.[9,10] Prophylactic administration of the compound following myocardial infarction was reported to reduce the incidence of ventricular arrhythmias as well as the occurrence of reinfarction during the hospital stay.[11] Mexilitine (Ko 1173, 2) and procainamide were compared in a 12 day study in a group of acute myocardial infarction patients.[12] Both drugs suppressed arrhythmias but the former exerted fewer side effects. Mexilitine was also evaluated in an extended study of 1 to 16 months.[13] Satisfactory control of arrhythmias was achieved in 79% of patients. Side effects included tremors, dizziness, nausea and blurred vision, although the authors concluded that the drug was well tolerated in most patients. A recent symposium detailed the efficacy of aprindine (3) in ventricular and supraventricular arrhythmias.[14] The drug suppressed the former in particular. Troublesome neurological side effects occurred in some patients.



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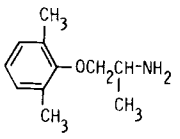

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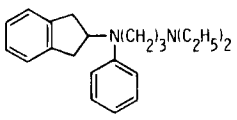
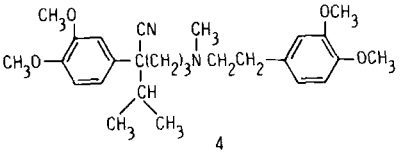


3

In a recent study intravenous verapamil (4), an agent which inhibits transmembrane influx of calcium, effectively suppressed supraventricular tachycardias but proved much less beneficial in ventricular tachycardias.[15]

The orally active lidocaine analog tocainide (W-36095, 5) suppressed premature ventricular contractions following single doses as well as during several days of therapy.[16,17] Although the compound was generally well tolerated, side effects of central nervous system origin appeared in some patients at blood levels not greatly above the therapeutic range.



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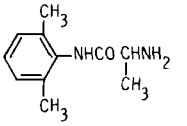
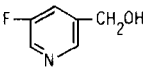


5

Plasma free fatty acids (FFA) are elevated during the early phase of myocardial infarction. Rapid reduction of FFA following the administration of the nicotinic acid analog 5-fluoro-3 hydroxymethylpyridine hydrochloride (6) to patients with infarction was associated with decreased incidence of ventricular arrhythmias.[18] Amiodarone (7) was reported to be effective in the treatment of atrial and ventricular arrhythmias in a recent trial.[19]



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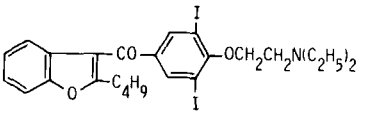
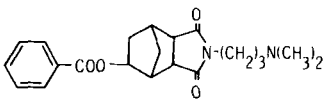


7

Newer antiarrhythmic agents - A comprehensive review of new agents has appeared recently.[20,21] Results of detailed experimental evaluation of several newer compounds were also published recently. BL-3677A (8) suppressed ventricular arrhythmias produced by ouabain in dogs and proved to be more potent than lidocaine.[22] It also antagonized ventricular arrhythmias occurring 24 hours after coronary artery occlusion in dogs. The compound possesses local anesthetic activity and briefly reduced blood pressure. MK-251 (9) antagonized ventricular arrhythmias following myocardial infarction produced by the intracoronary injection of a sclerosing agent or by coronary artery occlusion in dogs.[23] It increased the dose of digoxin required to induce cardiac dysrhythmias in cats. The compound is reported to lack significant hemodynamic effects.



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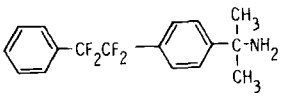

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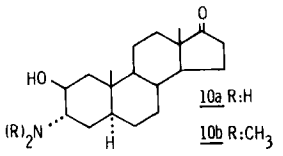
The aminosteroid ORG 6001 (10a) inhibited arrhythmias produced by aconitine in rats and by ouabain and coronary artery occlusion in dogs.[24,25] The compound also reduced the biochemical changes resulting from myocardial

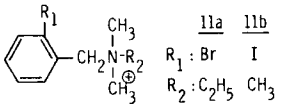


10a R:H

10b R:CH3

ischemia. ORG 6001 has a tendency to depress myocardial contractile force. Its dimethyl analog (ORG NA 13, 10b) also exerted antiarrhythmic activity but could not suppress conduction disturbances produced by ouabain.[26]

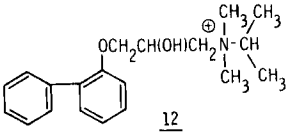
Interest in the antiarrhythmic activity of quaternary ammonium compounds continues. Unlike bretylium (11a), its o-iodobenzyl trimethylammonium analog (UM-360, 11b) did not inhibit release of norepinephrine from sympathetic nerve endings and did not cause adrenergic stimulation on its own.[27] UM-360 effectively antagonized ventricular arrhythmias produced by ouabain in dogs, an action bretylium did not possess. Both agents elevated the threshold to electrically induced ventricular fibrillation. The same group of workers also studied the profile of a quaternary propan-2-ol derivative (UM-424, 12).[28] In contrast to tertiary compounds of



11a 11b

R1 : Br I

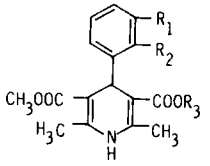
R2 : C2H5 CH3



12

generally similar structure, UM-424 did not block cardiac β-adrenergic receptors. The compound suppressed ventricular arrhythmias produced by ouabain and coronary occlusion and elevated ventricular fibrillatory threshold. It produced only brief depression of blood pressure and cardiac contractility.

The potential role of the calcium current in the genesis of arrhythmias was discussed above. Verapamil (4), which inhibits calcium influx, can suppress experimental arrhythmias.[21,29,30] Another calcium blocker, nifedipine (13a), inhibits irregular rhythms produced by calcium in isolated atrial preparations.[31] The calcium antagonist TMB-6(14) was reported to be as effective as lidocaine in suppressing digoxin-induced arrhythmias in dogs.[32] As expected, inhibition of calcium influx leads to depression of cardiac contractility and atrioventricular conduction.

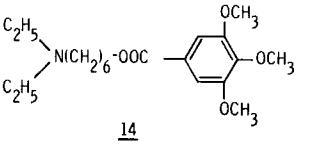


13a 13b

R1 : H NO_2

R2 : NO_2 H

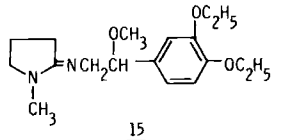
R3 : CH_3 $(CH_2)_2N(CH_3)CH_2C_6H_5$



14

The antiarrhythmic activity of newer β-adrenergic blocking agents was reviewed recently.[20,29,30] Most β-blockers exhibit at least some degree of antiarrhythmic activity since catecholamines contribute to the development of experimental and, particularly, clinical arrhythmias.[1,3,5] In addition, some of these agents affect the conduction system directly.[1,2,7,8,20] However, considerable quantitative as well as qualitative differences exist in regard to antiarrhythmic efficacy.

McN-2840-46 (15) suppressed experimental atrial arrhythmias preferentially in dogs.[33] Higher doses were required to antagonize conduction disturbances following coronary occlusion. MJ-9067 (16) antagonizes ventricular arrhythmias in dogs and exerts membrane depressant activity in isolated Purkinje fibers.[34,35]



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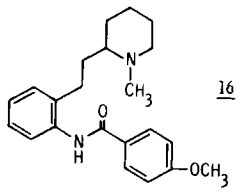

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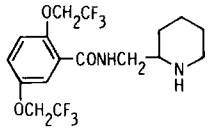
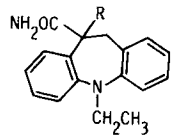


17

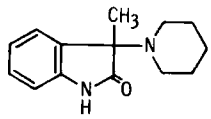
R-818 (17) prevented chloroform-induced ventricular fibrillation in mice as well as arrhythmias caused by hydrocarbon-epinephrine, ouabain, aconitine and coronary occlusion in dogs.[36] The activity of numerous analogs in the mouse chloroform test has been described.[37]

A series of dibenzazepines (18a, 18b) were compared to disopyramide (1).[38] The compounds suppressed ventricular arrhythmias in dogs but may have been more toxic than the latter. Preliminary reports on a series of 3-amino-3-methyloxindoles indicate that one member (19) is as effective as lidocaine in the mouse chloroform test and possesses a higher LD_{50}.[39]



18a R: H

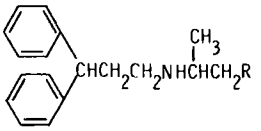
18b R: $(CH_2)_2N(CH_3)CH_2C_6H_5$



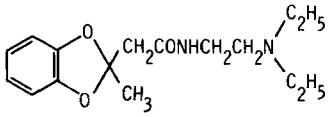
19

MG 8926 (20a), a substance closely related to prenylamine (20b), antagonized ventricular arrhythmias which followed abrupt occlusion of a coronary artery in dogs as well as conduction disturbances produced by ouabain in guinea pigs. In general, prenylamine exerted similar effects.[40]

A group of 2, 2-disubstituted-1,3-benzodioxoles were evaluated for their ability to inhibit arrhythmias in rats resulting from the i.v. injection of calcium chloride. Compound 21 was effective and possessed a relatively high therapeutic ratio.[41]



20a R: C_6H_{11}
20b R: C_6H_5



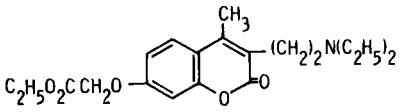
21

Antianginal Agents

The major determinants of cardiac oxygen consumption are heart rate, myocardial wall tension (directly related to ventricular cavity pressure and volume) and force and velocity of contraction. Under normal conditions, the heart extracts oxygen at a near-maximal rate and added requirements are met by a reduction in coronary vascular resistance and an increase in blood flow. In the atherosclerotic heart, coronary vessels in the ischemic region and particularly in the subendocarium are already dilated and their ability to increase flow by further dilatation is severely compromised. Thus, under these conditions, increasing oxygen demand may exceed supply, myocardial ischemia ensues and the patient manifests symptoms of angina pectoris.[42]

Development of novel antianginal therapy has suffered from the lack of predictive animal models. Some of the more interesting newer approaches include direct measurement of myocardial oxygen tension,[43,44] electrocardiographic analysis in normal[45,46] and diseased hearts,[47] regional vascular resistance studies,[48] blood flow distribution[49,50] and functional evaluation in normal hearts[51] as well as in animals subjected to chronic coronary artery occlusion.[52,53] The clinical predictability of these models remains undetermined.

Clinical reports - Organic nitrates and the β-adrenergic receptor blocking agents constitute the mainstay of therapy.[42,54-58] The mechanism of action of nitroglycerin remains uncertain. Although recent evidence tends to underline its peripheral vascular activity,[54,55] several studies have also implicated a direct coronary artery or collateral response.[43,46,52,59,60] Clinical data generally support the hypothesis that primary coronary vasodilators are of limited value in this disease since vessels in ischemic areas are already dilated.[42] However, beneficial results have been reported in patients with the vasodilators chromonar (22)[61] and lidoflazine (23).[62,63]



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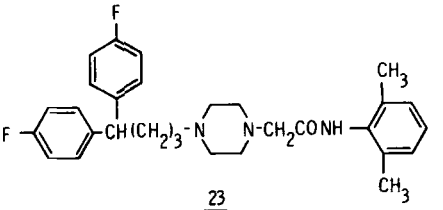

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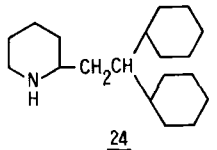
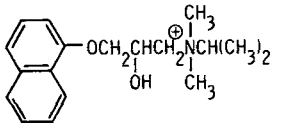


24

Perhexilene (24) continues to demonstrate antianginal efficacy.[64,65] Although the compound dilates coronary vessels, other mechanisms such as a reduction in exercise tachycardia and myocardial flow redistribution may be responsible for its effectiveness.[66,67]

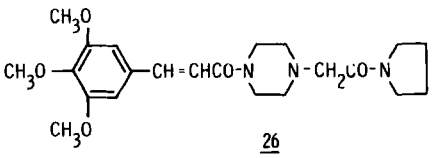
Favorable clinical results have been reported recently with the calcium antagonists nifedipine (13a)[68,69] and verapamil (4).[70] Again, although these drugs increase coronary blood flow experimentally, their clinical effects probably result from other mechanisms. Nifedipine can reduce myocardial contractility and oxygen consumption following intracoronary injection in dogs and can promote collateral development.[68,71,72] An analog (YC-93, 13b) produces marked vasodilatation in animals.[73] Prenylamine (20b) has also been reported to reduce the frequency of anginal episodes.[74]

Newer experimental agents - The dimethyl quaternary analog of propranolol, UM-272 (SC-27761, 25), a non beta-blocker, has been shown to reduce myocardial oxygen consumption and infarct size in dogs, presumably via a non-specific reduction in heart rate and contractility.[75,76]



25

The cardiovascular effects of a new "benign" coronary vasodilator, cinepazide (26), were recently reported.[77,78] The compound increased perfusion of ischemic areas in the dog heart. Specific coronary vasodilation was demonstrated in the dog heart with a new chromonar derivative morocromen (27).[79] Coronary activity was also noted in humans following oral administration.[79]



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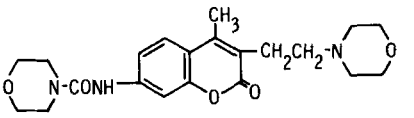
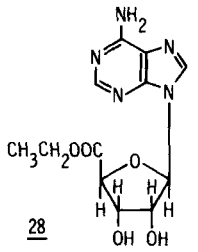


27

The adenosine derivative, Abbott 40557 (28) produced sustained coronary vasodilatation in dogs.[80] A reduction in feline myocardial oxygen consumption was reported with the Russian antianginal agent, chloracyzine (29).[81]

The prenylamine analog MG 8926 (20a) inhibited electrocardiographic manifestations of cardiac ischemia, i.e., S-T segment elevation, in dogs following coronary artery occlusion.[40]



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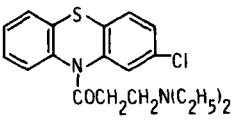

29

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Chapter 6 Cerebral Vasodilators

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Introduction

Physiology of cerebral blood flow - Despite fluctuations in systemic blood pressure, blood flow to the brain is maintained within very narrow limits by a process known as autoregulation[1]. This involves changes in the caliber of the cerebral vessels and thus in cerebrovascular resistance which are brought about by a variety of neurogenic and humoral factors[2-4]. It is now generally believed that the caliber of larger extraparenchymal vessels is controlled by nerve tone alone, whilst that of the smaller intraparenchymal vessels is controlled by both nerve tone and by local prevailing metabolic conditions such as tissue CO_2, O_2 and H^+ concentrations[3,5,6]. Innervation of the extraparenchymal vessels is by sympathetic fibers originating from the superior cervical ganglion and by parasympathetic fibers carried in the facial nerve[2,4]. Adrenergic brain stem neurones are considered solely responsible for innervation of the intraparenchymal vessels[6,7]. In vivo and in vitro studies with catecholamines have shown that α-adrenergic agonists cause cerebral vasoconstriction whilst β-adrenergic agonists cause vasodilatation[8,9,10]. In addition it has been suggested that physiological activation of the parasympathetic component of the innervation to the extraparenchymal vessels also results in vasodilatation[10-12]. As well as these adrenergic and cholinergic mechanisms, it is possible that the vasodilatory polypeptide (VIP) recently identified in cerebrovascular nerves also plays an important part in the regulation of cerebral blood flow[13].

Pathological changes in cerebral blood flow - A reduction in regional blood flow has been recorded in several acute and chronic cerebral diseases. The acute conditions include apoplexy, intracranial tumors, head trauma, hypoxia, compression of the brain, highly increased intracranial pressure and cerebral infarction[14,15]. Autoregulation is usually severely impaired because of vasomotor paralysis which results from the extreme tissue acidosis prevailing in each of these conditions[14]. Cerebral blood flow is also reduced in a variety of chronic diseases such as senile and presenile dementia[16-18], schizophrenia[19], and cerebral arteriosclerosis[20]. The exact cause of the reduced regional cerebral blood flow in dementia and schizophrenia is not clear, but may be related to a primary decrease in local neuronal metabolism[18,21]. In the case of cerebrovascular sclerosis the reduction is related to the occlusive nature of the lesions and to the reduced reactivity of cerebral blood vessels to physiological stimuli[22].

Clinical aspects of cerebral vasodilator therapy - Although cerebral vasodilators normally induce increases in cerebral blood flow under physiological circumstances, clinical results are often disappointing. Sometimes even a paradoxical reduction in the flow rate through a diseased brain area is recorded, or alternatively, the drug-induced increase in perfusion may be far in excess of the tissues metabolic requirements. These phenomena, known as the 'Intracranial steal phenomenon' and the 'luxury perfusion syndrome' respectively[23] are important complicating factors in the treatment of cerebral ischaemia. The therapeutic rationale of prescribing vasodila-

tor drugs in the light of such observations has recently been critically reviewed[24-28]. It was generally concluded that those compounds with a primary vascular action (i.e. those classified as vasotropic dilators below) seldom prove to be of substantial clinical benefit.

Methodology for testing potential cerebral vasodilators - Methods for the pharmacological and clinical testing of cerebrovascular active substances have been discussed in several recent reviews[26,29,30] and symposia[18,31,32]. The ability to detect changes in local cerebral blood flow related to increased neuronal activity and glucose metabolism is a particularly important recent methodological advance[33]. Various books[22,34,35] and symposia proceedings[18,31,32,36-40] dealing with many aspects of cerebrovascular circulation have recently been published.

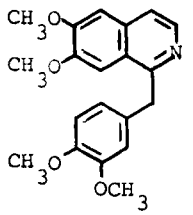
Specific Cerebrovasodilating Agents

Basically two groups of cerebrovasodilators can be identified according to their site of action. In the first group are those which have primary vascular action, dilatation usually being effected either by a direct relaxant action of the drug on the smooth muscle cells in arteriolar walls or by an inhibitory effect on the endogenous vasoconstrictive nerve fibers. A majority of these agents have been developed initially as peripheral vasodilators which have then been tested for their ability to increase cerebral blood flow on quite empirical grounds. The second group contains those which stimulate neuronal metabolism, the resultant increase in local perivascular CO_2 production consequently causing vasodilatation. The former group will be referred to as 'Vasotropic dilators' and the latter as 'Cerebrometabolic stimulants'.

(A) Vasotropic dilators

Chemistry - Of the five compounds discussed in detail below the first, papaverine, is a long recognized alkaloid of the benzylisoquinoline group which was originally isolated from opium. The remaining four compounds are all synthetic. There are no striking structural similarities between any members of this group which could indicate a structure activity relationship.

1. Papaverine - Papaverine (1) is a non-specific smooth muscle relaxant.



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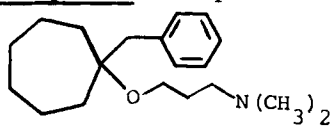
This action probably relates to its potent ability to inhibit phosphodiesterases[41,42]. By relaxing vascular smooth muscle and thus causing vasodilatation, papaverine increases mean cerebral blood flow in both animals[26,43,44] and man[45,46]. Whether papaverine produces useful clinical improvements in conditions of decreased cerebral blood flow is nevertheless debatable. Most studies have involved long term papaverine administration to geriatric patients suffering from 'chronic brain syndrome' due to longstanding cerebrovascular insufficiency. In four double blind studies papaverine was shown to improve various neurological and psychological symptoms[47-50], whilst in two further studies no such beneficial effects could be demonstrated[51,52]. Papaverine has not

been widely employed in the treatment of acute cerebrovascular disorders, but preliminary studies seem to suggest that it may have beneficial action in the treatment of certain sequelae[53,54]. The reported side effects of papaverine therapy are typical of those encountered with peripheral vasodilatory agents generally.

2. Bencyclane - The pharmacological profile of bencyclane (2) has received comprehensive coverage[57,58]. Basically bencyclane possesses direct musculotropic spasmolytic effects like papaverine, although it does not inhibit phosphodiesterases[58]. Bencyclane may block the uptake of calcium ions into vascular smooth muscle cells[58], thereby dilating both peripheral and cerebral vessels and consequently increasing cerebral blood flow in animals[59,60] and man[57,61]. Whether bencyclane is really effective in increasing cerebral blood flow in situations where flow is pathologically reduced is still questionable[62,63]. Bencyclane also inhibits platelet aggregation both *in vivo* and *in vitro*, although it is not certain whether this effect occurs at the dosages currently used clinically[57,64-67]. In a double blind study bencyclane was shown to improve symptoms related to acute cerebral ischaemia[67], whilst in two further studies no drug related improvement could be demonstrated[57,68]. Studies in patients with chronic cerebrovascular insufficiency have also yielded conflicting results. In a series of double-blind studies, bencyclane either improved the symptoms[57,69-72] or failed to have a clear effect[57,68,73]. This inconsistency is rather difficult to understand since dosage, duration of medication and the target symptoms studied were often very similar. The negative inotropic and chronotropic effects of bencyclane on the heart and the prolongation of the refractory period of heart muscle cells may be related to effects on calcium fluxes[74].



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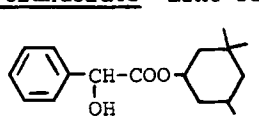
3. Cyclandelate - Like both papaverine and bencyclane, cyclandelate (3) has direct smooth muscle relaxant properties which account for its potent vasodilating effects[75]. Its exact mechanism of action is still not clear. On the basis of its known effects on the peripheral circulation, clinical studies were instigated to test for a possible cerebrovasodilating action. The results of a double blind study showed cyclandelate both to increase brain blood volume and to rectify pathologically prolonged circulation times[76]. These results were later confirmed by measurements of cerebral blood flow employing ^{133}Xe clearance techniques[77]. An early double blind study demonstrated both a significant decrease in mean cerebral circulation time and an improvement in the mental function of elderly patients receiving cyclandelate compared with those administered placebo[78]. Five similar double blind studies on geriatric patients have subsequently yielded extremely variable results[79-83]. Those conducted over longer periods were the more successful in showing a drug-related improvement and it has therefore been suggested that cyclandelate may prove more effective in the prophylaxis of progressive mental decline than in its treatment.



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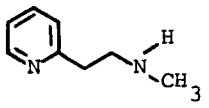
4. Betahistine - Betahistine is an orally active histamine analogue (4).



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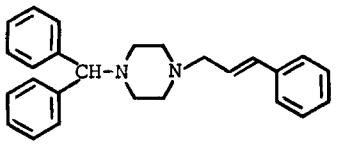
Its actions on peripheral and cerebral vessels are thus similar to those of histamine and can be antagonized by antihistaminic compounds[84]. Its predominant vasoaction is enhancement of the microcirculation, presumably by a relaxant effect on the smooth muscle cells of the precapillary arterioles. The molecular mechanism by which histamine itself induces such effects is not fully elucidated, but may involve a lowering of the intracellular ionized calcium concentration[85]. Unlike histamine, the vascular actions of betahistine are long lasting and it does not markedly stimulate gastric secretion[84]. Betahistine increases cerebral microcirculation, thereby increasing mean cerebral blood flow in both animals[86] and man[87,88]. Recent clinical results have been variable and often conflicting. In one double blind cross-over study involving patients with dementia related to vertebrobasilar insufficiency, betahistine increased both mean and regional blood flow values and improved the neurological and psychological status of the patients[89]. In a second study, again involving patients with cerebrobasilar insufficiency, but where transient ischaemic attacks were the target symptoms investigated, there was no such clear drug-related benefit[90]. Yet another study seems to suggest that patients experiencing vertigo due to cerebrobasilar insufficiency derive benefit from betahistine[91].

5. Cinnarizine - Cinnarizine (5) increases cerebral blood flow in both man[92] and animals[93] by causing cerebrovasodilatation[94]. The mechanism of action of cinnarizine relates to its ability to inhibit depolarization-dependent calcium uptake into arterial smooth muscle[95]. Conversely the release of calcium ions from bound intracellular stores within the muscle fibres by noradenaline is unaffected. This suggests that cinnarizine specifically blocks calcium uptake at the outer membrane, thereby preventing or reducing muscle tension and so producing vasodilatation. Clinical results with cinnarizine, as with the other vasotropic dilators, have been variable. In three double blind studies conducted on patients with cerebral arteriosclerosis, medication produced a clear-cut improvement in their clinical status[92,93,96]. In a fourth study where cinnarizine and digoxin were given together, the combination produced considerably better results than cinnarizine alone[97]. In contrast to the foregoing, a multilocational study performed in Britain failed to demonstrate any beneficial action of cinnarizine in patients with longstanding cerebral arteriosclerosis[98].



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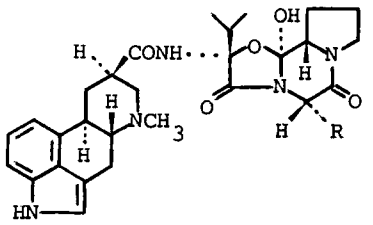
(B) Cerebrometabolic stimulants

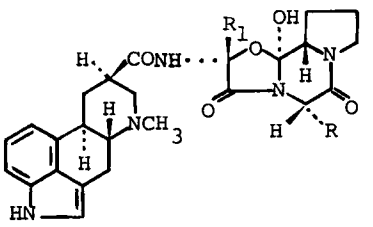
Chemistry - All the well established, clinically active compounds in this category are naturally occurring, or derived from naturally occurring ergot and vincamine alkaloids. Although both chemical groups are the subject of intense chemical investigation and molecular manipulation no structure-activity relationships for their cerebrometabolic stimulating properties have yet been published.

1. Hydergine ® - Hydergine ® (6) contains dihydroergotoxine mesylate as the active principle, which is an association of the mesylates of dihydroergocornine(6a), dihydroergocristine(6b), dihydro-α-ergocryptine(6c), and dihydro-β-ergocryptine (6d) in the ratio 3:3:2:1 (ref.99). Hydergine® possesses α-adrenergic blocking activity and thus induces some degree of vasodilatation by inhibiting the action of noradrenaline tonically released from nerve endings within vessel walls. However, the greater part of its overall peripheral vasodilating action probably results from a central inhibitory action on the vasomotor center[100,101]. Under conditions of experimentally reduced cerebral blood flow Hydergine® induces dilatation of the brain capillaries thereby lowering vascular resistance and improving both blood flow and glucose utilization[102]. The mechanism by which Hydergine® effects this is probably quite different from that involved in its dilatation of the peripheral cirulation. Based on recent experimental results it has been suggested that Hydergine® primarily protects neuronal metabolism under conditions of inadequate cerebral perfusion[44,102]. The observed increase in blood flow is thus considered entirely secondary to the local increases in CO_2 production that accompany improved neuronal metabolism[102-105].



6 a R = $CH_2C_6H_5$
b R = $CH(CH_3)_2$
c R = $CH_2CH(CH_3)_2$
d R = $CH(CH_3)CH_2CH_3$

That the increased blood flow is a consequence of effects on neuronal metabolism and not the cause of them is further supported by the observation that Hydergine® increases the reduced EEG activity occurring in both animals and man with pathologically reduced cerebral blood flow. Simply correcting the flow rate by cerebrovasodilatation with papaverine does not produce such an effect[106,107]. The exact mechanism by which Hydergine® stimulates neuronal metabolism is not clear, but may relate to its ability to activate dopamine receptors in the pontomedullary reticular formation or to its central serotonergic properties[108,109]. Preliminary studies with the semi-synthetic dihydrogenated ergot alkaloids dihydroergonine (DN 16-457, 7) and dihydro-β-ergosin (DQ 27-422, 8) suggest that these compounds may possess cerebral stimulating properties similar to those of Hydergine® [108]. Recent studies on its molecular mechanism of action suggest an important effect of Hydergine® on neuronal cAMP levels[110]. By inhibiting a low K_m-phosphodiesterase, Hydergine® may prevent neuronal cAMP concentrations from falling excessively in certain pathological situations. Conversely both in slices of rat cerebral cortex and in cat brain homogenates, norepinephrine-induced increases in cAMP levels are antagonized by low concentrations of Hydergine®, probably through its ability to block the α-adrenergic effects of norepinephrine at the post synaptic membrane.

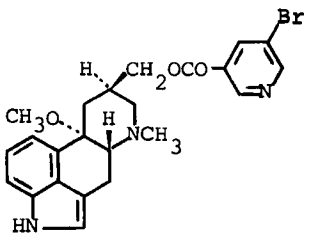


	R	R_1
7	C_2H_5	$CH(CH_3)_2$
8	CH_3	$CH(CH_3)CH_2CH_3$

Hydergine ® also partially inhibits norepinephrine-induced activation of neuronal Na^+-K^+ ATPase by a similar anti-adrenergic mechanism[111].

Hydergine® effectively decreases cerebral circulation times in patients with prolonged values and increases both cerebral oxygen utilization and CO_2 production[104,112,113]. Several recent double blind studies have demonstrated a useful action of Hydergine® in the treatment of senile dementia. In three such studies Hydergine® was shown to be significantly better than placebo in ameliorating many of the neurological and psychological symptoms[114-116]. In two further studies conducted over a twelve week period, it was possible to correlate such improvements in the patients' mental status with drug-related changes in their EEG patterns[107,117]. In five of the most recent studies comparisons with papaverine have been made. In each case Hydergine ® has been found to be superior for the treatment of the various mental manifestations studied. In these studies Hydergine® ameliorated such symptoms as confusion, irritability and depression, whilst mental alertness and motivation were consistently improved[51,118-121]. The improvements in intellectual function and cognition were always among the most striking. Hydergine ® also had a beneficial effect on certain physical symptoms such as fatigue, dizziness, noctural cramps and anorexia.

2. Nicergoline - Nicergoline (9) is an ester of 10α-methoxy-dihydrolysergol.



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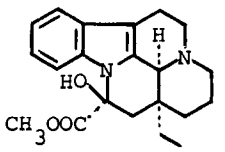
It possesses considerable α-adrenergic blocking action and consequently induces dilatation of the peripheral microvasculature[122]. Like Hydergine® , it increases cerebral microcirculation by primary protective effects on neuronal metabolism[123]. Under conditions of experimental ischaemia, nicergoline increases both neuronal glucose uptake and reduces pyruvate and lactate formation[124]. In animals these effects are reflected in a more rapid post ischaemic recovery of EEG activity[125]. Its molecular mechanism of action seems related to effects on brain ATP levels, which are more rapidly restored after experimental ischaemia when pretreatment with nicergoline has been performed[126]. Like Hydergine® and dihydroergotamine, nicergoline stimulates cAMP accumulation in rat cerebral cortex slices by an inhibitory action on phosphodiesterase. Part of this rise in cAMP may be mediated via a direct stimulation of adenylate cyclase[127]. Nicergoline also increases cerebral oxygen and glucose consumption in elderly patients suffering from cerebral arteriosclerosis[128]. In one study it was claimed that nicergoline increases cerebral blood flow and the symptoms associated with acute or chronic reduction of brain perfusion rates[129]. However this must still remain conjectural since, although another study has confirmed a drug-induced increase in cerebral blood flow in patients with recent apoplexy, no such increase was detected in those with chronic or diffuse cerebrovascular pathology[130]. No carefully controlled clinical studies with nicergoline have yet been reported. In one open, uncontrolled study, fifteen elderly patients suffering from cerebral arteriosclerosis were considered to have benefited from nicergoline therapy[131].

3. Vincamine - Vincamine (10) is a plant alkaloid, which has been shown to increase cerebral blood flow in both man[61] and animals[132]. Recent animal studies suggest that vincamine stimulates neuronal metabolism, thereby increasing glucose utilization and CO_2 production. The resulting rise in pCO_2 of the perivascular neurones is thus believed to be the definitive vasodilating stimulus[133]. There are very few recent, well documented clinical studies with vincamine. In one double blind study conducted on geriatric patients suffering advanced cerebral arteriosclerosis, vincamine apparently improved the EEG pattern as well as the associated neurological symptoms[134]. In a second study it was claimed that a redistribution of cerebral blood flow to pathologically affected areas accompanied such improvements in clinical status[135].



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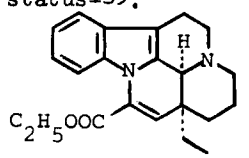

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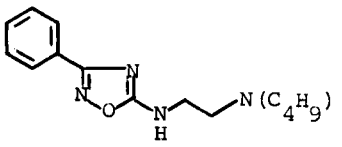
Recently the chemical, pharmacological and clinical aspects of a new vincamine analogue have been reported (Cavinton® RGH-4405, 11)[136]. It is a simple ester of apovincaminate. This agent appears to possess useful clinical action for the treatment of various cerebrovascular disorders.

(c) Miscellaneous Compounds

Butalamine (12) is a compound which induces vasodilatation by relaxing vascular smooth muscle and which seems promising clinically[137]. Dextran, a polysaccharide which improves the flow qualities of the blood by a volume expansion effect, and should not perhaps be considered a true cerebral vasodilator, is nevertheless still one of the most efficacious compounds for the treatment of acute cerebral ischaemia[138]. Fenoxedil (13) seems to induce cerebral vasodilatation by both vasculotropic and cerebrometabolic actions[139,140]. Isoxsuprine (14) is an adrenaline derivative with pure β-adrenergic stimulating action. It causes cerebral vasodilatation by this mechanism and improves various symptoms related to decreased cerebral perfusion[141]. Moxisylate (15) is a specific α-adrenergic antagonist with beneficial clinical action[142]. Nafronyl oxalate (16) possesses cerebral metabolic stimulant activity and thereby brings about cerebral vasodilatation[143]. Nicotinyl alcohol (17) is a primary cerebrometabolic stimulant which improves clinical symptoms associated with decreased cerebral blood flow[144]. Pentoxifylline (18), like theophylline (19) possesses phosphodiesterase inhibiting action. Both compounds relax vascular smooth muscle by this mechanism and thus cause vasodilatation. They may also have effects on neuronal metabolism[145-147]. Piracetam (20) is a primary cerebrometabolic stimulant with claimed efficacy in the treatment of senile dementia[148]. Proxazole (21) is papaverine-like in action. It causes increases in cerebral blood flow[149] and improves the symptoms of cerebrovascular insufficiency[150]. Raubasine (22) is an α-adrenergic blocking agent and possibly possesses neurometabolic stimulating properties. In patients with reduced cerebral blood flow due to arteriosclerosis, raubasine increases blood flow and improves their clinical status[151,152]. Viquidil (23), increases cerebral blood flow by inducing vasodilation in a similar way to papaverine[153]. Hexobendine (24) is a cerebrometabolic stimulant that increases cerebral blood flow[154].



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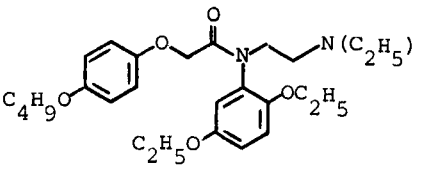

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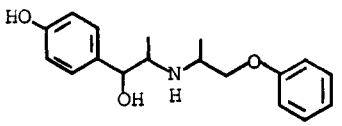

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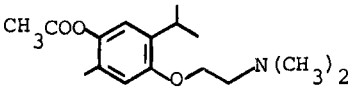

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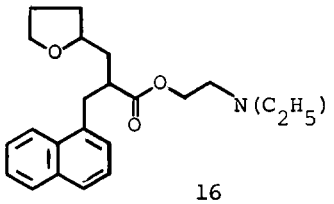

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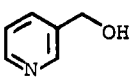

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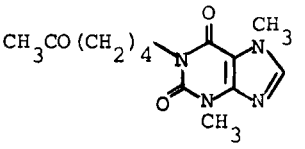

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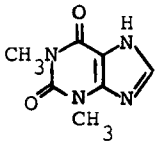

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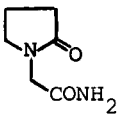

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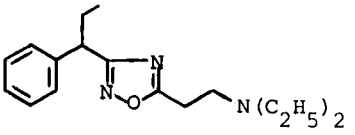

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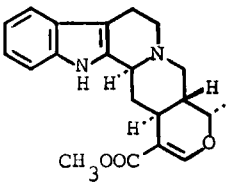

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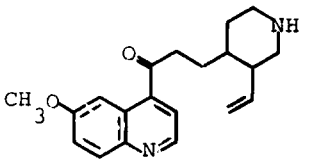

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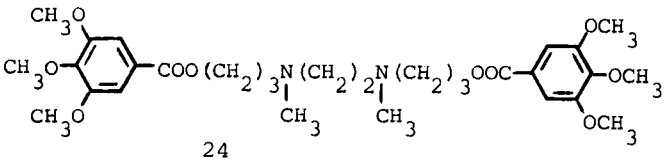

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Chapter 7. Antihypertensive Agents

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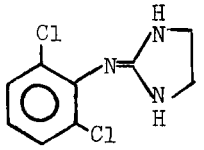
Introduction - Reviews have been published on the physiology of cardiovascular regulation[1] and the biochemical etiology of hypertension.[2] It has been proposed that hypertension results from a repeated sequence of small rises in blood pressure, due to sympathetic hyperactivity, which produce changes in the kidney.[3] The roles of cadmium, lead[4] and catecholamines[5] in the development of hypertension and the cardiovascular and renal actions of prostaglandins[6] have been reviewed. The drugs used in the clinical management of hypertension have been surveyed.[7]

Central Mechanisms - A useful review of the physiology of central cardiovascular regulation has appeared[1] and two reviews[8,9] deal concisely with the central noradrenergic control of blood pressure. Less well-understood aspects of the pharmacology of central control, including the central effects of angiotensin, are dealt with in a congress report.[10]

Clonidine (1)(Boehringer-Ingelheim) has continued to attract attention. A study[11] of 22 congeners of clonidine has correlated the peripheral α-stimulant properties with physicochemical parameters; however, the central hypotensive action using i.v. or intracerebroventricular (i.c.v.) dosing, could not be so correlated. Indeed, the nature of the central receptors and their relationship to peripheral α-receptors remains unclear. It has been suggested[12] that clonidine may activate central H_2 receptors, since i.c.v. metiamide, a specific H_2-blocker, was able to reverse and antagonize the hypotensive but not the bradycardiac effect of i.v. clonidine in anaesthetized rats. Clonidine was also shown[13] to stimulate at relatively high concentration an H_2-receptor-coupled adenylate cyclase in the hippocampus of guinea pigs. However, there is no evidence to suggest a histaminergic action of clonidine in conscious hypertensive cats.[14] (Histamine itself given centrally is pressor in conscious cats via an H_1 mechanism[15]). The striking finding that clonidine acts purely as an antagonist to noradrenaline stimulated increases in cAMP in rat brain slices has been amplified.[16] A comprehensive study in rats[17] of the functional and biochemical actions of clonidine and the effects of different antagonists has given further support to the developing concept that pre-synaptic α-stimulation is involved in some responses. The central sympatho-inhibitory effect, however, is probably a post-synaptic action.[18] A novel technique involving the measurement of electrodermal responses to demonstrate clonidine-induced central sympathetic inhibition has been described.[19] The water diuresis produced in conscious dogs by clonidine appears to be consequent upon an increase in renal PGE synthesis, which has an anti-ADH (antidiuretic hormone) effect.[20] The ability of very low doses of clonidine to reduce vascular reactivity has again been demonstrated.[21] A well-described clinical investigation of the effects of 300 μg p.o. clonidine in normotensive volunteers has given useful data on the sedation and hyposalivation side effects against which similar agents

might be compared.[22] The first clinical investigation of the hypertensive rebound phenomenon on abrupt cessation of clonidine therapy failed to find evidence of the effect in a small number of mild-to-moderate hypertensives after short term treatment.[23]

Clinical data on BS-100-141 (2)(Sandoz) are now readily available.[24,25] The compound is as effective an antihypertensive in man as clonidine but ten times less potent. Sedation and dry mouth appeared with the same frequency as with clonidine. The central α-stimulant action of guanabenz (3) in cats appears distinct from that of clonidine in that baroreceptor mechanisms are not involved in the hypotensive response.[26] In the clinic, guanabenz (24-48mg) produced modest blood pressure reduction with sedation as the major side-effect.[27]



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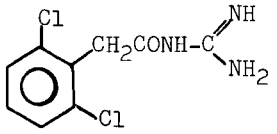

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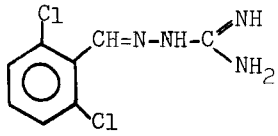

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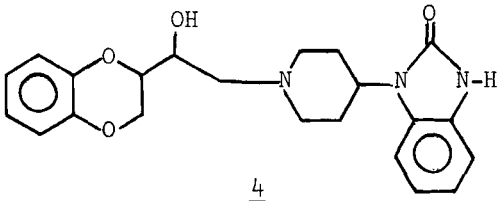
Central hypotensive mechanisms not involving α-stimulation remain ill-defined. Janssen R28935 (4) was last year's major development in this area and the suggestion has been made recently that central α-blockade is responsible for the hypotensive response to intracisternal injection in rabbits of R28935, BE2254 (5) and phentolamine.[28]



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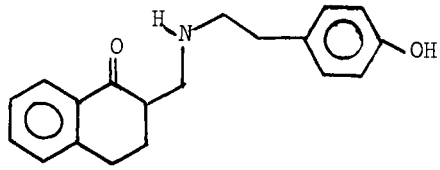

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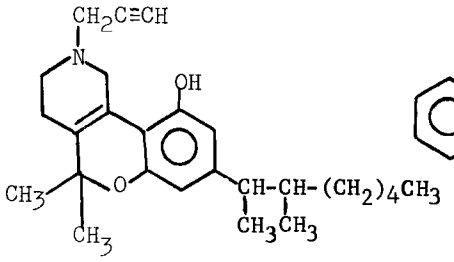
The mechanism of the clinical hypotensive action of β-blockers remains contentious, but several papers[29] have demonstrated further the ability of centrally-administered blockers to lower blood pressure in experimental animals. It is not certain that this effect results from β-blockade, however.

Further evidence that central 5-hydroxytryptamine (5-HT) neurones are involved in cardiovascular control systems is found in the observation that a central administration of the selective neurotoxin 5,6-dihydroxytryptamine causes a significant delay in the development of hypertension in young spontaneously hypertensive rats (SHRs).[30] A further demonstration of the centrally-mediated hypotensive effect of methysergide has appeared;[31] this may not be a result of 5-HT antagonism.

Tetrahydrocannabinol is considered a lead for novel centrally-acting hypotensives. Cardiovascular activity was found frequently in a wide-

ranging series of variations of the cannabinoid structure.[32] As an example, 6 produced long-lasting hypotension with bradycardia upon oral administration to SHRs and neurogenically hypertensive dogs.



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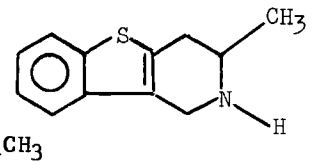

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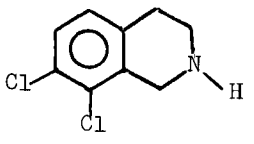
Levels of phenylethanolamine-N-methyltransferase (PNMT) have been found to be significantly raised in discrete brain stem regions in both SHR and DOCA-salt hypertensive rats.[33] Administration of SKF 7698 (7), a PNMT inhibitor, normalized pressure in DOCA rats. The possibility that the effect was due to the adrenolytic action of (7) was not excluded. The best of a new series of PNMT inhibitors with much reduced α-blocking properties is SKF 64139 (8).[34]

In two hypertensive rat models, whole brain tyrosine hydroxylase levels correlated well with systolic blood pressure.[35] The hypertensive response to the phosphodiesterase inhibitor RA-642 (9) administered in the vertebral artery to cats originates in the medulla oblongata[36] and is a result of activation of the sympathetic outflow. Conversely, a preliminary report[37] suggests that central administration of a phosphodiesterase activator such as imidazole lowers blood pressure.



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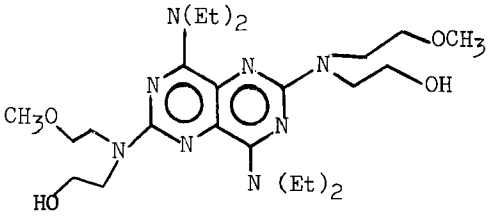


9

The Renin-Angiotensin System - The regulation of renin release,[38,39] its biochemistry,[40] clinical significance[39,41] and the role of the renin-angiotensin system in extra-renal tissue[42] have been reviewed. The effects of antihypertensive agents on the renin-angiotensin system have been surveyed.[43] Evidence has been obtained for a renal α-adrenergic receptor inhibiting renin release in rats[44] and man.[45] Dopamine and apomorphine induce renin secretion; the effect of apomorphine is blocked by pimozide.[46] In man,[47] rats and rabbits[48] plasma renin activity is suppressed by indomethacin concomitantly with a reduction in prostaglandin synthesis. In

rats and rabbits arachidonic acid stimulates plasma renin activity.[48] Lysophosphatidylethanolamine derivatives inhibit renin release in rats.[49] Low renin essential hypertension has been found to be associated with a suppression of sympathetic nervous activity.[50]

Angiotensin converting enzyme has been reviewed.[51] It is a single peptide chain associated with polysaccharide and contains a zinc atom. On the basis of a suggestion that the active site of this enzyme may resemble that of carboxypeptidase A, novel orally active angiotensin converting enzyme inhibitors have been designed such as 3-mercapto-2-**D**-methyl-propanoyl-L-proline (Squibb 14,225).[52] This compound has a marked and sustained antihypertensive effect on renal hypertensive rats at doses of 3 to 10mg/kg. A new enzyme, tonin, has been described which can produce angiotensin II (AII) from AI. Its activity appears to be under the influence of β-adrenergic receptors.[53] The structure activity relations of peptide antagonists of the renin-angiotensin system such as substrate analogues, converting enzyme inhibitors and angiotensin II antiagonists have been reviewed,[54] and the clinical significance of angiotensin blockade has been surveyed.[55]

Beta-Adrenergic Blocking Agents (β-Blockers) - Studies on the identification and characterisation of β-adrenergic receptors have been reviewed.[56] β-Receptors have been identified in the rat brain[57] and localised using a fluorescent β-blocker.[58] The clinical pharmacology of β-blockers has been summarised.[59] The use of propranolol (ICI) (10) in hypertension has been reviewed.[60] It is now available in the U.S.A. for this indication.[61a] Atenolol (ICI 66082, 'Tenormin') (11) has been launched[61b] in the U.K. for the treatment of hypertension.[62] It is a cardioselective β-blocker without intrinsic sympathomimetic activity. Its duration of activity allows once a day dosing.[63] Bunitrolol (Ko 1366 'Stresson')(12) a **cardioselective** β-blocker has been marketed in Austria by Boehringer-Ingelheim.[64] The medicinal chemistry of the compounds related to acebutolol (13)(May and Baker) has been described[65] and the first clinical report of talinolol (14)(Veb Arzneimittelwerke Dresden) a cardioselective agent has appeared.[66]

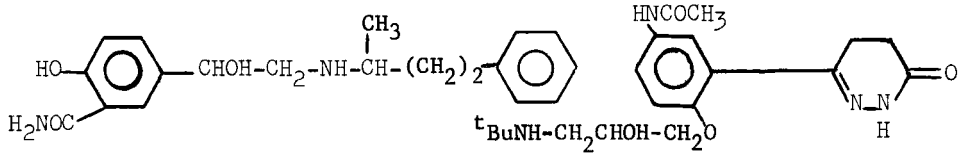
$ArOCH_2CHOHCH_2NHR$

		Ar	R
10	Propranolol	1-naphthyl	iPr
11	Atenolol	4-H_2NCOCH_2-phenyl	iPr
12	Bunitrolol	2-cyanophenyl	tBu
13	Acebutolol	4-butyramido-2-acetyl-phenyl	iPr
14	Talinolol	4-[3-cyclohexylureido-]-phenyl	tBu
15		2-[3-cyanopyridyl]	iPr

Although a large number of β-blockers have been investigated in the clinic, their mode of action in hypertension is still unknown.[60] It is unclear whether all agents act by the same or the same combination of effects and whether all the observed effects are due to β-adrenergic receptor antagonism. The role of renin continues[67] to be argued without **new** conclusions.[68] The central depressor effects have been reviewed.[69] It has

been suggested that damping sensory input to the CNS from the heart may diminish sympathetic nerve efferent activity.[70] β-Blockers may act by controlling the surges in blood pressure resulting from stress.[71] The effects of β-blockers on adrenergic transmission have been discussed.[72a] It has been proposed that β-blockers may act by reducing noradrenaline release by blocking presynaptic β-receptors mediating positive feedback.[72b] Presynaptic effects have also been described for prostaglandins, dopamine, histamine, nicotine and angiotensin.[73]

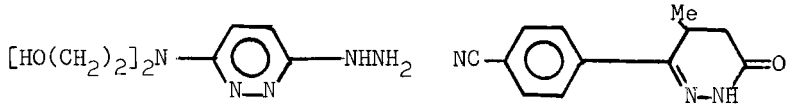
In addition to their common use in combination with vasodilators and diuretics,[74] β-blockers have been used with α-adrenergic blockers[75] although it might be argued[76] that this combination would give a similar effect to adrenergic neurone blockers. Labetalol (Allen and Hanbury 5158) (16) is a non-selective β-blocker with weak α-adrenergic blocking properties.[77] It is 4-8 times more potent at the β than at the α-receptor. In man it causes a rapid fall in blood pressure with reduced peripheral resistance but little change in cardiac output. The relative effects of the α- and β-blockade appear to vary with the dose used.[76] Postural hypotension is noted particularly at higher doses but is said to be reduced



16 17

with chronic treatment. It could be a useful agent in hypertensive emergencies and in patients with moderate hypertension who do not respond to β-blockers and diuretics. Compounds with β-blocking activity and vasodilating properties such as 15[78a] and 17[78b] have been patented.

Antihypertensive Vasodilators - Prazosin[67] (Pfizer) has been introduced[61c] into the U.S. Unlike other vasodilators it causes a decrease in plasma renin levels.[79] The use of hydralazine has been reviewed,[80] and the structure-activity relations of hydrazinopyridazines and phthalazines have been studied using molecular orbital calculations[81] and Hansch analysis.[82] Animal pharmacology has been summarised and the first clinical data reported[83] for Lepetit 6150 (18). In 20 patients it was found to be 8 times



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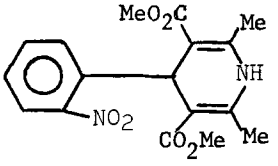
more potent than hydralazine in lowering diastolic blood pressure but only 2-4 times more potent in raising heart rate. CL90,394 (Lederle)(19) has a long duration of action in rats, lowering blood pressure with tachycardia at 10 mg/kg p.o. The vasodilation and tachycardia are partially blocked by propranolol.[84] No α-adrenergic, neurone or ganglion blockade was observed and the compound did not inhibit MAO, tyrosine hydroxylase or dopade-

carboxylase. Bupicomide (5-butylpicolinamide)(Schering 10,595) reduced systolic and diastolic blood pressure in 6 out of 10 patients. Peripheral resistance fell and there was an increase in heart rate.[85] There is evidence in rats that fusaric acid (5-butylpicolinic acid) the main metabolite of bupicomide, causes tachycardia by indirect release of catecholamines.[86]

Nifedipine ('Adalat', Bayer 1040)(20), used for the treatment of angina,[87] is a powerful peripheral vasodilator and has hypotensive properties in man.[88] A dose of 30mg sublingually lowered blood pressure substantially in 14 essential hypertensives for more than 4 hrs. with a rise in heart rate and plasma renin activity. Nifedipine interferes with the transmembrane calcium flux causing a reduction in vascular smooth muscle tone.[87,89] The conformational requirement for dopamine induced renal



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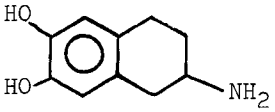
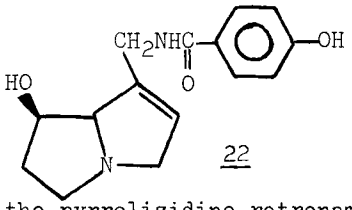


21

vasodilation has been identified[90] as the fully extended form related to 21. The mode of action of organic nitrates as vasodilators has been reviewed.[91] Some amides of adenosine-5'-carboxylic acid lower blood pressure in SHRs. They may act directly on an adenosine receptor.[92] Some vasodilators may act as adenosine deaminase inhibitors.[93]

Miscellaneous Agents - A number of bisbenzyltetrahydroisoquinolines from Thalictrum species have been found to lower blood pressure in dogs.[94]



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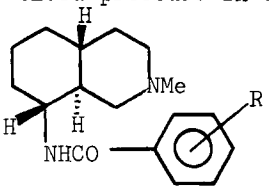

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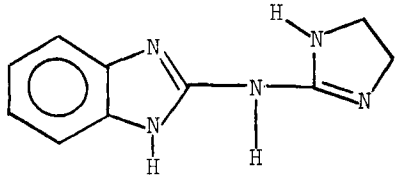
Amides of the pyrrolizidine retronamine, such as 22, have been found to be hypotensive[95] and the decahydroisoquinolines 23 lower blood pressure in SHRs.[96] A new antihypertensive dopamine-β-hydroxylase inhibitor, BRL-8242 (24) has been described.[97] In metacorticoid and normotensive rats, the hypotensive response to oral administration was dose-related in the range 3-100 mg/kg and was shown to correlate with the reduction of endogenous noradrenaline in the tissues examined. Heart rate was not changed significantly. BRL-8242 was shown to have Cu^{++}-complexing properties, in vitro.



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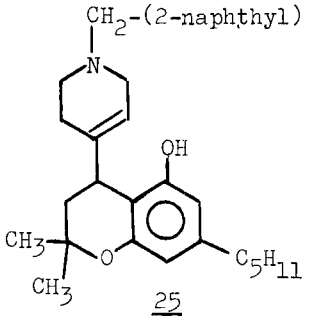

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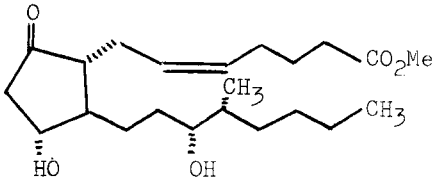
Another Beecham compound, BRL 13776 (25) has shown antihypertensive properties due to noradrenaline depletion in DOCA rats and renal hypertensive cats.[98] Only the medulla/pons region of brain showed significant drops in noradrenaline, in contrast to reserpine. There were no behavioural effects and the compound is being taken to clinical trial. A novel hypotensive peptide, hypotensin, has been isolated from the venom of the Western diamondback rattlesnake.[99] It contains approximately 20 amino-acid residues and appears unrelated to the kinins. The hypotensive effect is said to be dose-related after oral administration in normal rats and SHRs and is not consequent upon histamine release. Brief details of a clinical trial of a PGE_2 analogue (26) are available.[100] Eleven of seventeen hypertensives responded with lower blood pressure to oral doses (10-20 μg) of 26. A recent study[101] of the marked antihypertensive properties of the diuretic Indapamide (27)(SE 1520, Servier) in rats and cats shows it to reduce vascular reactivity on chronic dosing.



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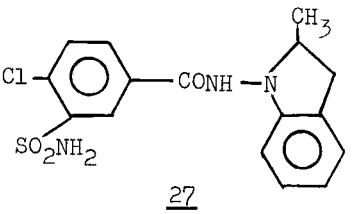

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Chapter 8. Pulmonary and Anti-Allergy Drugs

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General – Work in the area of immediate hypersensitivity has continued to define the mechanism of mediator release stimulation by the interaction of antigen with specific cell bound immunoglobulin. Characterization of specific mediators such as slow reacting substance of anaphylaxis (SRS-A), eosinophil chemotactic factor of anaphylaxis (ECF-A) and histamine have appeared.[1-5]

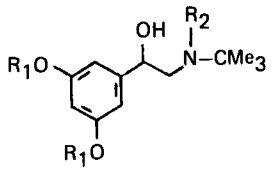
The interaction of cell bound immunoglobulins IgE with antigen and haptens ultimately leads to the release of mediators of immediate hypersensitivity from these stimulated cells. IgE appears to interact with a specific plasma membrane receptor on the target cell. Characterization of the cellular receptor for IgE was determined using rat peritoneal mast cells and rat basophilic leukemia cells and indicates it has a molecular weight of 60,000.[1] The suggestion was made that this molecule is a single polypeptide chain. Experiments defining the nature of the cell surface IgE receptor will significantly contribute to the understanding of antigen antibody complex induced release of mediators of allergy and biochemical modifications of this process can now be envisioned. Pharmacologic studies have indicated that cyclic adenosine 3',5'-monophosphate (cAMP) modulates mast cell and basophil secretion.[2] Agents that increase cAMP levels usually inhibit mediator release, whereas agents that decrease cAMP enhance release. However, that concept may undergo some modification since rat mast cells challenged with anti IgE antibody or Conconavalin A indicated that histamine release from these cells was preceded by increases in cAMP levels.[2] Therefore, cAMP modulation of mediator release appears to be more complex than initially discerned. Eosinophil chemotactic factor of anaphylaxis (ECF-A) is a mediator of immediate hypersensitivity reactions and has been characterized. ECF-A activity resides in two acidic tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu and synthetic tetrapeptides with these sequences were preferentially chemotactic for eosinophils.[3] Histamine also modulates human eosinophil migration by interacting with H_1 and H_2 receptors. At higher histamine concentration ($> 10^{-5}$M) H_2 receptor interactions predominate producing inhibition of eosinophil movement. At 10^{-6}M or less, H_1 receptor activation by histamine resulted in enhanced eosinophil migration.[4]

Another mediator of the allergic reaction, SRS-A, thought to be present only in basophils, has now been shown to be present in human leukocytes as well,[5] from which it was released by stimulation with calcium ionophore A23187. Until now, allergic mediators in primate systems have been derived from tissue mast cells or basophils. Since these mediators also reside in other cell types and may be released by non-immunologic mechanisms, this suggests that these agents may play a more general role in inflammatory processes.[5]

Clinical – In a recent study total secretory immunoglobin A (IgA) and specific anti-antigen E were measured in nasal washings from ragweed allergic and normal individuals.[6] None of the data support the hypothesis that allergic individuals are deficient in secretory antibody responses but the data do support the concept that hay fever sufferers belong to a high responder population which genetically responds to low doses of inhalant antigens.[6] Other studies have shown that IgE antibodies (usually thought to induce only immediate skin reactions) in combination with appropriate antigen on the surfaces of mast cells or infiltrating basophils cause both immediate and late cutaneous responses.[7] These skin responses may be analogous to late onset asthmatic responses induced by inhalation of ragweed and house dust, etc. This technique might serve as a valuable tool in analyzing the anti-asthmatic effects of new pharmacologic agents.

Techniques for the diagnosis of anaphylactic sensitivity to hymenoptera (bees, wasps, ants, etc.) stings have been difficult to establish. Studies with commercially available whole body extracts of hymenoptera for skin testing were unable to discriminate between hypersensitive and control subjects. Use of hymenoptera venom skin tests clearly distinguishes between allergic and normal subjects[8] and this venom was made available to the National Institute of Allergy and Infectious Diseases for further uses as a diagnostic material in 1976.[8]

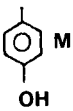
Beta-Adrenoreceptor Stimulants – The development of β-stimulant bronchodilators, their pharmacology, evaluation, metabolism and structure activity relationships has been reviewed.[9] The goal is still an orally effective compound which is a specific β-2 adrenoreceptor stimulant with a long duration of action. Terbutaline (1) was marketed in the U.S. in 1974 and has been studied extensively. It is effective by inhalation (0.50 mg)[10] and orally (5 mg)[11] in the prevention of exercise-induced bronchospasm and its bronchodilating effect is evident for 6 hrs. Orally, peak effect is 2-3 hrs; by inhalation, 5-30 min.[12] 1 (0.25 mg/s.c.) is effective in the management of acute bronchospasm in stable asthmatics.[13] Several long term studies of 1, both oral (5 mg t.i.d.) for 32 months[14] and by inhalation (375 μg t.i.d. for 6 weeks)[15] showed good efficacy with no drug tolerance[16] and only minimal side effects such as occasional tremor.[14] Metabolism studies on ^{3}H-terbutaline indicate sulfate and glucuronide conjugation whether administered by i.p. or by inhalation.[17] Studies on 1 and its prodrug, Ibuterol (2), show that after 2 the serum concentration of 1 rose more rapidly (30 min.) and to higher levels (4.1 μg/ml) than after 1 itself.[18] Structure activity studies indicate β-2 selectivity is greatest when the amine moiety is t-butylamine (terbutaline) or p-hydroxy N-t-butylaniline (ME-106) (3), as determined by cat soleus muscle, bronchi and heart rate experiments.[19]



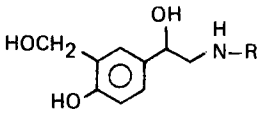
1. R_1=H
2. R_1= 
3. R_1=H

R_2=H terbutaline

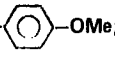
R_2=H ibuterol

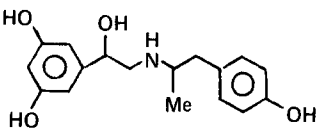
R_2=  ME-106

Salbutamol (4) has been extensively studied by oral (4 mg), inhalation (200 μg),[20] intravenous,[21] and intramuscular[22] administration and found to be effective in the inhibition of exercise induced bronchospasm.[20] No drug tolerance was observed in chronically pre-treated guinea pigs.[23]



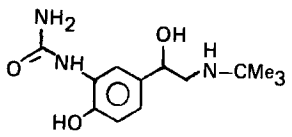
4. R=t-butyl; salbutamol

5. R=CH(Me)CH$_2$–  –OMe; salmefamol.

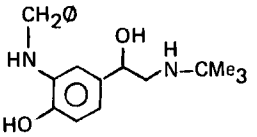


6. fenoterol (Th 1165a)

In patients with chronic airways obstruction salmefamol (5) gave greater response than 4, particularly 6 to 8 hr. post dose.[24,25] Orally (2 mg) 5 caused a fall in diastolic blood pressure, whereas 1 mg dose did not, and both were found equally effective on ventilatory capacity.[26] By inhalation fenoterol (6) was equipotent with 4 in the treatment of asthmatic children but caused some increases in pulse rate.[27] Orally, 6 caused significant bronchodilatation with rapid onset (30 min.) and long duration (6 hrs.) but showed mild tremors and increased pulse rates at higher doses (15, 20 mg).[28] Carbuterol (7) (SKF 40383) in a double-blind study produced safe and effective bronchodilatation both orally (4 mg) and by inhalation[29] (300 μg), but was less potent than 4.[30]



7. carbuterol (SKF 40383)



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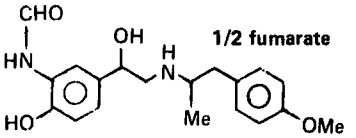
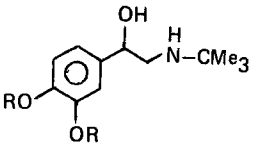


1/2 fumarate

9. BD-40A

A series of compounds of general structure (8) variously substituted in the benzyl group were claimed to be 10 x 4 in potency as bronchodilators with higher bronchoselectivity (cat soleus muscle prep).[31] BD 40A, (9) is more potent than 4 by various routes but is less β2-selective.[32]

A series of mono and diesters of N-t-butylarterenol (10) showed that the monoesters have a moderate degree of



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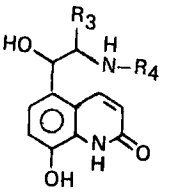
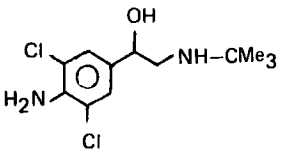


11

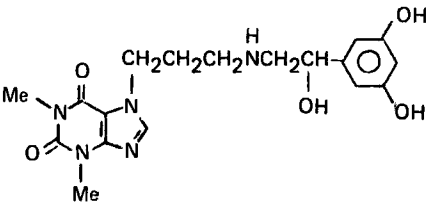
activity with rapid onset and a duration of action of 2 hrs. The diesters show marked bronchodilatation by i.v., i.d. and inhalation administration with a 5-10 min. onset of action and lasting 4 hrs. The diester of choice is 4-Me-C_6H_4-CO-(Bitolterol).[33] Hydrolysis rates of the esters correlate well with the bronchodilatator activity seen in intact, anaesthetized dogs.[33] Bitolterol is less cardiostimulating than 4 in dogs at doses which cause some bronchodilatation.[34]

Structure activity relationships for a series of sympathomimetic amines having a carbostyril moiety (11) have been reported.[35] The most potent (11, R_3=H, R_4=t-butyl) is 22,400 x isoproterenol in relaxation of guinea pig trachea, and is also more β-2 selective than 4. Order of activity: R_3: H>Me>Et, R_4: t-butyl>i-Pr>$CMe_2CH_2\emptyset$>Bz>H.[35] Another analog (11, R_3=Et, R_4=$CHMe_2$) (OPC 2009) is claimed to be more active and more selective than 4.[36]

Detailed pharmacology, toxicology, pharmacokinetics and metabolite patterns of clenbuterol (12), (NAB-35) in rat, rabbit, dog and man have been reported.[37] A double-blind study has shown 12 orally effective (10 μg t.i.d.) in the treatment of moderately severe asthma.[38]



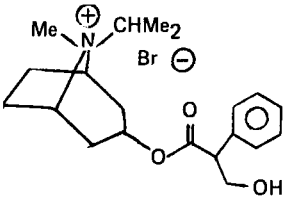
12. clenbuterol (NAB-35)



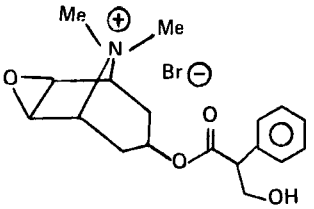
13. reproterol (D 1959)

Detailed accounts have been published of the synthesis, structure activity relationships and pharmacology of a series of bronchospasmolytic β-phenylethylaminoalkylxanthines, of which (13) (reproterol, D 1959) was selected for clinical trial. 13 is a β-2 agonist with minimal CNS or CV side effects and is clinically effective against bronchial asthma and chronic bronchitis (orally or by inhalation) with no tachyphylaxis after 4 weeks.[39]

Anticholinergics – Although the precise role of anticholinergics in the treatment of asthma is not yet known, they have been advocated as an alternative to β-agonist therapy in patients with cardiac arrhythmias or angina.[40] Ipratropium bromide (14) (Sch. 1000, Atrovent®) by inhalation showed bronchodilator activity comparable with isoproterenol, but had a longer duration of action (4 hr.) with no significant side effects.[41]



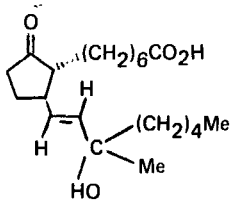
14. Sch 1000
Atrovent®



15. Pamine®

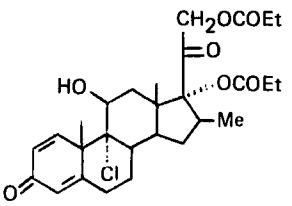
Side effects of 14 appear minimal, i.e., dry mouth observed at maximum doses in some patients.[42] 14 appears to have bronchospasmolytic action and no secretory inhibition properties at therapeutic doses, and is without effect on sputum viscosity or volume.[42] Detailed studies on the synthesis, general pharmacology and toxicology in rats, dogs, mice and monkeys by various routes of administration of 14 have been published.[42] Pharmacokinetic and metabolism studies of 14 show a t 1/2 i.v. of 1.9 hr. (rat), 3.4 hr. (dog) and 4 metabolites have been isolated and characterized.[42] Inhalation of atropine (0.1 mg) blocked exercise induced bronchospasm and caused significant bronchodilatation for up to 5 hr.[43] Studies conducted in 1949 on methscopolamine (Pamine®) (15) in which prolonged efficacy against the bronchospastic effects of methacholine in asthmatic patients was noted were restated. No cardiovascular side effects but drying of the oropharynx were observed.[44]

Prostaglandins – The pharmacologic actions of prostaglandins and their role in allergy has been reviewed.[45] Inhalation of $PGF_{2\alpha}$ in normal subjects produces 2 qualitatively different airway responses, which may reflect the balance between sympathetic and parasympathetic nervous control of the airways. Diminished β-receptor activity in asthmatic patients may account for heightened bronchoconstrictor response to $PGF_{2\alpha}$.[46] Plasma levels of 15-keto-13,14-dihydro $F_{2\alpha}$ (a relatively stable metabolite of $PGF_{2\alpha}$) were measured under various conditions in exercise induced asthmatics, but did not show any significant change, leading to the conclusion that $PGF_{2\alpha}$ does not play a significant role in the aetiology of exercise induced asthma.[47] The contractile effects of some cycloendoperoxides (CEP's), intermediates in the biosynthesis of PG's, are 3 to 4 orders of magnitude greater than PGE_2 and $PGF_{2\alpha}$ and may represent the active form of PG's in the lung.[48,49] The synthesis of ± 15-methyl-11-deoxy PGE_1 (doxaprost, AY-24,559) (16) and its C_{15} epimer have been reported.[50] 16, together with ± 11-deoxy PGE_1, inhibited histamine-induced bronchoconstriction in guinea pigs. 16 was 73 and 32 times more potent than 11-deoxy PGE_1 by the aerosol and i.v. routes, respectively. 16 also demonstrated a longer duration of effect.[51]

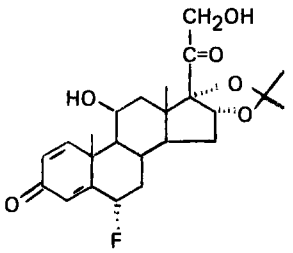


16. doxaprost, AY-24,559

Corticosteroids – Beclomethasone dipropionate (17) aerosol has been tested extensively clinically. It is used prophylactically (400 μg per day), not therapeutically, in the treatment of chronic asthma, particularly in children.[52] An evaluation of the drug has been published.[53] One of the most important clinical advantages is that 17 effectively can replace oral corticosteroids in steroid-dependent patients and avoid many of the adverse effects of adrenal suppression.[52,54] Most patients with impaired adrenal function due to oral corticosteroids show recovery of adrenal function within 6 months.[55] The combination of 17 and disodium cromoglycate (DSCG) showed no additive therapeutic effects.[56] Flunisolide (18), when administered as a nasal spray for 4 weeks during the hay fever season in 51 patients, showed significant symptomatic improvement with no systemic steroid effects observed.[57]

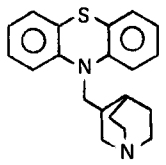


17. beclomethasone dipropionate

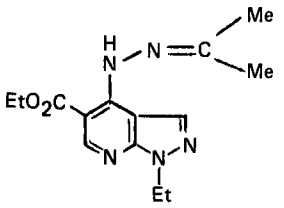


18. flunisolide

Phosphodiesterase Inhibitors – LM 209 (19) has been shown to be a noncompetitive inhibitor of lung phosphodiesterase (PDE) (2.5 x 10^{-4}M) in contrast to theophylline, which is a competitive inhibitor. 19 is antihistaminic, orally effective and inhibits bronchospasm provoked by histamine, serotonin or citric acid. It is distinguished from other antihistamines by its lack of sedation and long duration of action.[58] Absorption, distribution and elimination studies in rat and dog after i.v. or oral administration have been reported.[59]



19. LM 209



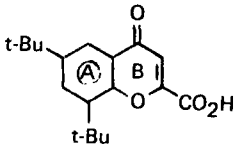
20. SQ 20,009

A pyrazolo[3,4-b]pyridine (20) (SQ 20,009), a potent inhibitor of cAMP PDE, inhibited antigen induced histamine release from passively sensitized guinea pig lung slices. 20 also inhibited the phosphatidylserine plus dextran induced release of histamine from rat peritoneal mast cells. DSCG and doxantrazole (30) also act as inhibitors in this system, but demonstrate cross-reactive tachyphylaxis. 20 shows no tachyphylaxis.[60] Serum concentration determinations are important in guiding safe usage of theophylline since a relationship between daily dosage and serum levels of the drug is not predictable.[61] Toxic symptoms occur commonly over levels of 25 μg/ml, but are not noted below 15 μg/ml.

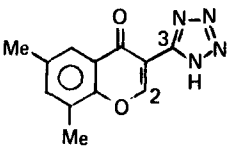
Inhibitors of Mediator Release – A review of several trials of disodium cromoglycate (DSCG) (Intal®) concluded that clinical benefit is obtained in 33-50% of patients.[63] Long term studies show that the early response to DSCG is maintained but not heightened over long periods of time (up to 5 yrs.).[64] The most valuable results of DSCG therapy are (a) a reduction in symptoms of asthma (sneezing and coughing), (b) no side effects, (c) reduced need for oral bronchodilators, aerosolized adrenergics and corticosteroids, and (d) greater exercise tolerance.[65] In a perennial allergic rhinitis trial it was found that patients which responded best were female and those who had high IgE levels and a markedly positive skin test to food and epidermoids rather than pollen allergy.[66] DSCG (orally) has been found effective in gastritis varioliformis[67] and ulcerative colitis.[68] After 10 months on DSCG, one patient developed pulmonary infiltrates with eosinophilia. The symptoms disappeared 2 weeks after the drug was withdrawn.[69]

Structure activity relationships for a series of chromone-2-carboxylic acids, of which FPL 52791 (21) showed efficacy in passive cutaneous anaphylaxis (PCA) (rat) both by intravenous and oral administration, have been published. Substitution in ring A shows that 6,8-di-t-butyl (12 x DSCG)>6,8-di-Et>6,8-di-Me>> monosubstitution.[70] Clinical studies of 21 are in progress. Similarly, in a series of chromones with a tetrazolyl moiety at the 2 or 3 position, (22) was the most potent in the PCA (rat), both orally and i.v. (~11.6 x DSCG). The following structure activity relationships were noted: (a) the 3-tetrazolyl

compound was twice the potency of the 2-tetrazolyl isomer, (b) introduction of a methyl group in position 2 of the chromone ring reduced activity, (c) substitution in the phenyl ring showed the following order of potency: 6,8-di-Me>6-Cl~6-Et>6-NO_2~6-OH. The naphthyl analogues, e.g., (23), were also active.[71]



21. FPL 52791



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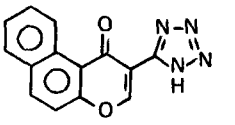
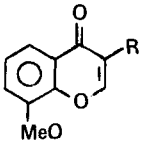
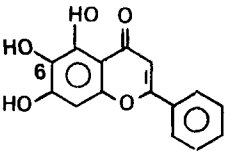


23

Metabolism studies of 24a (W8011) in rats identified 3 metabolites (24b, c, and 2-hydroxy-3-methoxyacetophenone). Although 24a and b are active *in vivo* (rat PCA), 24b was the only compound active in an *in vitro* system for inhibition of anaphylactic histamine release.[72]

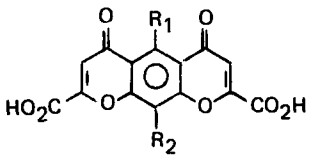


24a. R=CH_2OH (W8011)
b. R=CO_2H
c. R=H



25

The disodium-6-phosphate derivative of baicalein (25) inhibited PCA (rat) and experimental asthma caused by passive systemic anaphylaxis in guinea pigs.[73] A series of benzodipyrandicarboxylic acids (26) has been described and their structure activity relationships in PCA reported. Linear analogs 26 were more active than the corresponding angular analogs (27), although substitution effects differ from the linear to the angular series.



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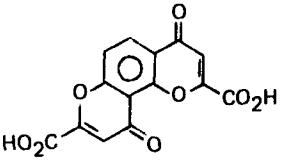

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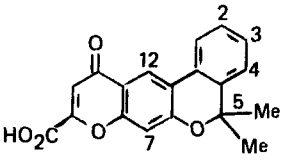
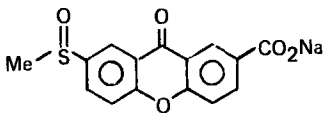


28. PRD-92-EA

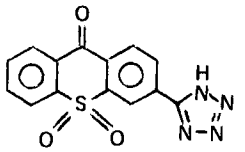
In 26, introduction of alkyl groups led to enhancement of activity, e.g., R_1, R_2=H: i.v. PCA 6 x DSCG; R_1=Me, R_2=Et: ~36 x DSCG, whereas introduction of alkoxy caused a reduction in activity. Conversely, in the angular series 27, introduction of alkyl substituents produced no significant change, but an alkoxy group ortho to a pyrone carbonyl group resulted in a marked increase in activity. Electron withdrawing substituents in either series resulted in loss of activity.[74]

Structure activity relationships for a series of compounds of which PR-D-92-EA (28) was the most potent (6 x DSCG) i.v. (rat PCA) have been published.[75] Simple substitution did not yield compounds with activity significantly enhanced over 28, with the exception of the 3-OCH_2CH_2OH,4-Me compound (~30 x DSCG).[75]

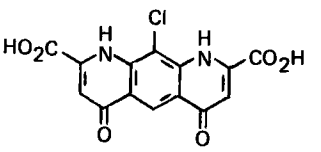
Clinical studies on tixanox[76] (29) (OTMX) by inhalation showed good protection which lasted up to 4 hr. in 5/6 patients with allergic asthma; 2 patients had a delayed (Type III) reaction 4-6 hrs. after antigen challenge which was unaffected by 29. No evidence of bronchodilator activity was observed.[77] Doxantrazole (30) is 10 x DSCG and 10 x theophylline as a PDE inhibitor, and the anti-allergic activity of 30 may be due in part to this ability to elevate intracellular levels of cAMP. Replacement of the tetrazole moiety by carboxyl results in a significant loss of activity (0.2 x 30).[78] The pharmacology of compound A, (31), indicates a qualitative and



29. tixanox (OTMX)

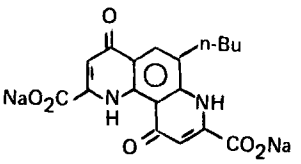


30. doxantrazole

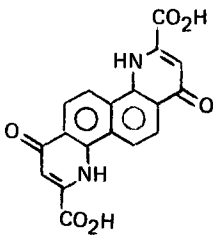


31. Compound A

quantitative advantage over DSCG in that (a) it shows bronchodilator activity, (b) inhibits mediator release (50 x DSCG), and (c) is orally effective.[79] On intrabronchial administration 31 inhibited the immediate type hypersensitivity to Ascaris antigen in rhesus monkeys (1000 x DSCG). 31 is presently in clinical trial.[80] Bufroline (32) (ICI-74917) is 5 x DSCG as a PDE inhibitor and 100 x DSCG in the PCA model.[81] Clinically, 32 by inhalation showed almost complete protection against nasal stenosis induced by grass pollen extract in 10 patients.[82] Studies on 32 and DSCG indicate that tachyphylaxis is related to the continued occupation of a receptor by drug molecules on the mast cell surface, preventing the access of further drug to these sites.[83] 32, DSCG and doxantrazole may act by interfering with calcium transport across the mast cell membrane.[84] The synthesis of ^{14}C labeled 32 has been described.[85] Of a series of compounds related to 32, 33 had comparable activity in the rat PCA.[86] In BM 15,100 (34) chemical and pharmacological combination of an antihistamine of the cyclizine type and anti-allergic agents of the DSCG type has been achieved.[87] 34 shows activity in rat PCA, both orally and by intravenous administration; it antagonizes bronchospasm in guinea pigs and prevents anaphylaxis in monkeys



32. ICI 74,917 (bufroline)



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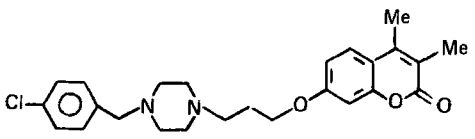
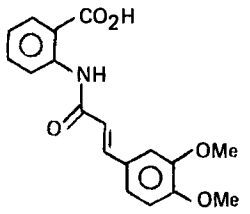


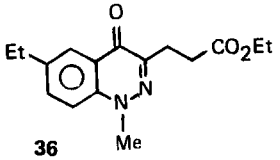
34. BM 15,100



35. N5[1]

induced by ascaris antigen.[87] An anthranilic acid derivative N5[1] (35) is orally effective in rat PCA and it is claimed to show good results clinically in the prophylactic treatment of asthmatic attacks.[88] Detailed pharmacological comparison of 35 with standard anti-inflammatory agents has been published.[89]

Structure activity relationships on (36) indicate that several cinnolone-3-propionic acid derivatives are comparable with DSCG in rat PCA, and 36 is also orally effective.[90] The esters are comparably active with the carboxylic acids (i.v.) due to rapid hydrolysis *in vivo*. 36 is not metabolized by β-oxidation to the



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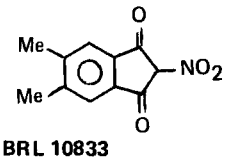
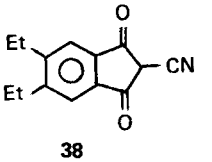


37. BRL 10833



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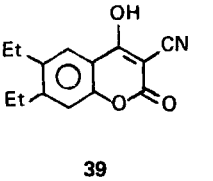
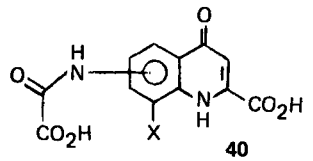


39

corresponding cinnolone carboxylic acid.[90] Inhalation or oral administration of the nitroindane dione BRL 10,833 (37) protected approximately 50% of patients against allergen-induced immediate bronchospasm and also showed some protection against the delayed bronchial reaction.[91] A number of 2-cyanoindane-1,3-diones (38) and 3-cyano-4-hydroxy coumarins (39) related to 37 have been reported active in rat PCA (i.v. and p.o.). The structural requirements for these new series parallel that already reported for 37.[92] Replacement of the hydroxy in 39 by a wide variety of groups resulted in loss of activity.

Structure activity relationships for a series of quinolyl oxamic acids (40) with several analogs showing good activity in rat PCA (25 x DSCG) have been published.[93] The most active compounds have the oxamic acid residue at the 6 or 7 position of the nucleus and for X: Me>Cl>MeO. The naphthyl analogue (41) showed



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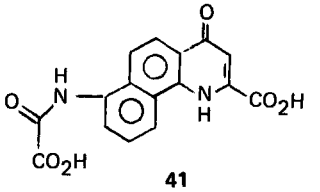

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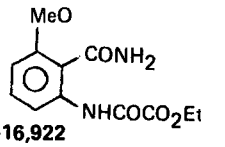
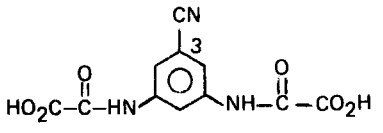


42. WY-16,922

comparable activity.[93] The N-aryl oxamic acid ester WY-16,922 (42) effectively inhibited reaginic mediated immunologic reactions in skin, lung and mast cell. It is more potent than DSCG and is orally active.[94] Structure activity relationships have been reported for a series of bisoxamic acids of which (43) showed high potency (250 x DSCG) in rat PCA i.v. and also oral activity. The order of activity for substituents at the 3 position of 43 is CN



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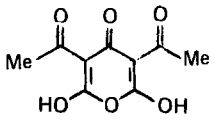

44

~NO_2>>OMe>$CONH_2$>>CO_2H.[86] Of a series of 3,5-disubstituted pyrantriones, (44) showed modest activity (0.05 x DSCG) in rat PCA.[95]

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Chapter 9. Antithrombotic Agents

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Introduction - Vascular diseases are the primary causes of death in western civilization. These diseases cause about 1 million deaths a year in the United States, with about 25 million people afflicted. Total costs of time lost and care exceeds 7 billion dollars per year. In over 90% of the vascular-related deaths, thrombosis was part of the death-related event. Though the chronic, insidious development of atherosclerosis is the main cause of the vascular diseases, thrombosis is the final death-producing event. Good reviews have been published on atherosclerosis, in which its development and interactions with the blood components are discussed.[1-6]

In order to prevent the acute events in vascular disease death, maintaining a physiologic balance between blood components, blood vessel wall, and circulatory function is important. Many approaches that may help control vascular disease are available. However, the interaction of the blood components with the diseased blood vessel wall, and the interaction of the blood components with each other must be considered as an important area to attack in the treatment of these diseases. In the search for weapons to help regulate the physiologic balance, antithrombotic agents should play an important role. Therefore development of new and potent antithrombotic agents would be an important contribution to the control of vascular disease.

Since the coagulation of blood is a complicated biochemical and biophysical process in which different blood components interact with each other and with the surrounding diseased environment, there can be multiple approaches to antithrombotic therapy.

The coagulation process has been reviewed.[7] The main approaches to its control are: 1. Control of fibrin formation. 2. Control of fibrinolysis activity, so that formed clots may be lysed. 3. Control of platelet function. Previous and more recent reviews on background in vascular disease, atherosclerosis, and on antithrombotic agents are recommended.[1-6,8,9] Previous reviews in this series[10-15] are also recommended.

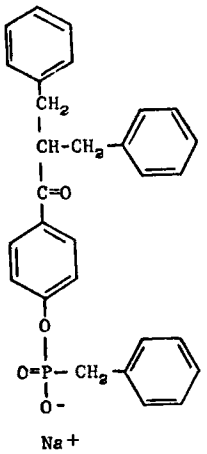
Platelet Aggregation Inhibitors

The role of platelets in vascular disease, both on theoretical and clinical bases, has been discussed in many reviews.[16-20] A report on ongoing clinical testing of aspirin and other inhibitors has also been summarized.[21] Clinical results with several of the older inhibitors (aspirin, sulfinpyrazone, dipyridamole, etc.) have been reported.[22]

These inhibitors are either not very potent, have side effects at therapeutic levels, and/or inhibit only one of several aggregation mechanisms. Adenosine diphosphate (ADP), thrombin, epinephrine, collagen, and serotonin induce platelet aggregation by different mechanisms. Compounds that specifically inhibit ADP-induced aggregation will not inhibit thrombin-induced aggregation. Those compounds that inhibit epinephrine and collagen-induced aggregation (e.g. nonsteroidal anti-inflammatory agents)

do not inhibit the primary phase of ADP or thrombin-induced aggregation. Therefore, combinations of specific agents for each type may be needed to give better control of in vivo platelet aggregation, or compounds that inhibit all systems should be considered. The following discussion has been organized with this in mind. Each type of compound is discussed in the mechanistic category that it fits, when this is known.

Aspirin and other Nonsteroidal Anti-inflammatory Compounds - The nonsteroidal anti-inflammatory compounds inhibit the production of labile aggregation-stimulating substances (LASS).[23] Most of them have been shown to inhibit the enzyme cyclooxygenase (synthetase) that produces the endoperoxides PGG_2 and PGH_2 from arachidonic acid (AA). These endoperoxides are intermediates in the synthesis of prostaglandins (PG) E_2 and $F_{2\alpha}$.[24] Recently Hamberg, Svensson, and Samuelsson[25] reported another series of reactions in which endoperoxide PGG_2 is a substrate. An enzyme found in platelets and smooth muscle converted the endoperoxides into 2 new fatty acid derivatives of which the first in sequence was very short lived (about 30 sec.) and was a very potent stimulator of platelet aggregation and smooth muscle contraction. This compound (1) is called Thromboxane A_2 ($TBXA_2$). $TBXA_2$ breaks down into Thromboxane B_2 ($TBXB_2$, originally called PHD)(2), which is much more stable (see Chapter 19). It is now believed that LASS as originally defined is $TBXA_2$. Since nonsteroidal anti-inflammatory compounds inhibit synthetase, they reduce formation of endoperoxides and therefore $TBXA_2$ and PG synthesis; this reduction affects smooth



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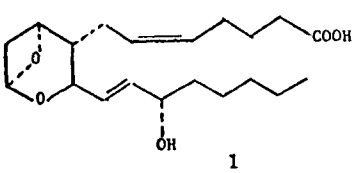

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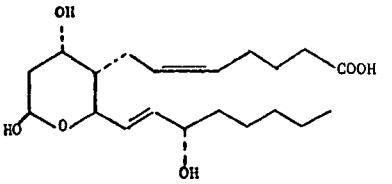

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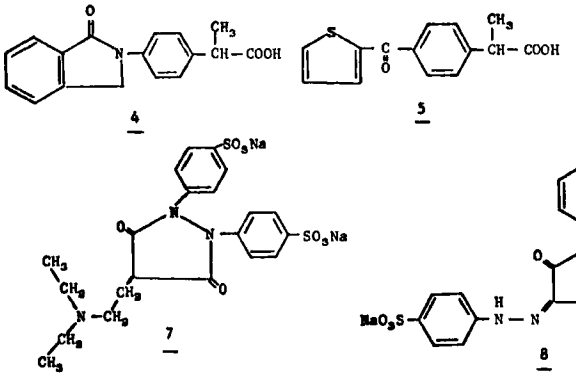
muscle contraction and the platelet "release reaction" and second phase platelet aggregation. This new important discovery makes it possible to look for compounds that will inhibit $TBXA_2$ formation, but will not inhibit PG synthesis. Such a compound has been reported, N-0164 (3).[26] It inhibited the formation of $TBXA_2$ from PGG_2 in vitro.

Of all the anti-inflammatory agents, aspirin is unique. It acetylates several proteins in the platelet. One protein in the particulate fraction, mol. wt. 75,000, is suggested to be that which is required for PGG_2 biosynthesis.[27] The effect of aspirin is irreversible. New normal platelets must be formed and enter the circulation to support the release reaction to the extent to cause second phase platelet aggregation (4-7 days). Other nonsteroidal anti-inflammatory agents have an effect only as long as they are present in the circulation (usually only 2-4 hours) and therefore must be given many times daily.

Aspirin has been widely investigated both preclinically and clinically.[28,29] Though there are many clinical studies in progress,[21] no concrete well-controlled results have as yet been completed and reported. The Aspirin/Stroke Study[30] conducted through a multi-center clinical investigation under the auspices of NIH has been finished and the data is being compiled. The results[31] indicate there is a statistically significant beneficial effect on the incidence of transit ischemic attacks (TIA, "little strokes"). In this study a relationship was sought between aspirin treatment, inhibition of platelet aggregation and prevention of TIA. A positive result would provide the first hand evidence for a clinically useful antithrombotic effect of aspirin. Though many physicians have prescribed aspirin for this type of effect, even a recommendation of 1 gram twice weekly for patients who have spontaneous platelet aggregation,[32] we still do not know which conditions would be best benefited by aspirin and which by other inhibitory agents.

Other previously reported nonsteroidal anti-inflammatory agents discussed in recent literature were phenylbutazone, sulfinpyrazone, and indomethacin.[33] Work on several previously reported propionic acid derivatives[34] was extended, and several new compounds have been reported to produce inhibition of platelet aggregation such as: indoprofen[35] (4) and suprofen[36] (5). Feprazone (6),[37] pyrazolidinedione 7,[38] and MYaK-38[38] (8) have structures more related to that of phenylbutazone.



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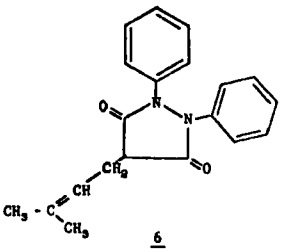

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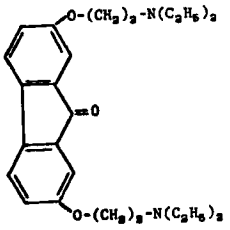

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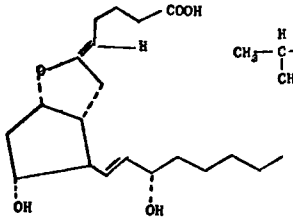
Tilorone (9) also has been reported to have anti-inflammatory activity,[39] and inhibits epinephrine and collagen-induced but not ADP-induced platelet aggregation.[40] Recently Ford-Hutcheson et al. reported that tilorone did not inhibit prostaglandin synthesis *in vitro* in the guinea pig lung system.[41] Thus tilorone may also affect the production or action of $TBXA_2$.

Prostaglandins and Synthetic Related Compounds - In past reviews[42,43] prostaglandins, natural and synthetic, have been discussed. Originally the rationale was to synthesize derivatives of PGE_1, then the most potent inhibitor of platelet aggregation known.[44] PGE_1 is active *in vivo* for only 5-20 minutes after infusion is stopped, and side effects limit its *in vivo* usefulness as a platelet aggre-

gation inhibitor. More recently, it has been reported that PGD_2 is an even better inhibitor.[45] Others have suggested that substitution in the diet of 5,8,11,14-eicosatetraynoic acid (TYA) for AA would produce inhibition of $TBXA_2$ synthesis and stimulate PGE_1 synthesis.[46] The inhibition of $TBXA_2$ and/or the increase in synthesis of PGE_1 or addition of a prostaglandin that inhibits platelet aggregation should help control thrombosis.

Recently it has been reported that a new prostaglandin called PGX or prostacyclin (10) is synthesized from PGG_2 or PGH_2 in the microsomal fraction of blood vessels.[47,48] This bicyclic prostaglandin is the most potent inhibitor of platelet aggregation found to date. J. Vane[48] speculates that when platelets bump into healthy endothelial cells, they release endoperoxides. The healthy endothelial cell releases an enzyme that converts the endoperoxides into PGX and prevents platelet aggregation. In areas of damaged endothelium this enzyme is absent, $TBXA_2$ is formed, and platelet aggregation occurs.



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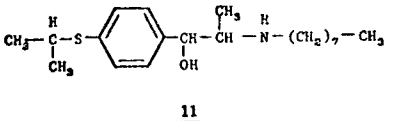

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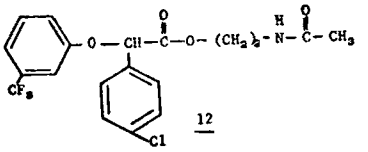

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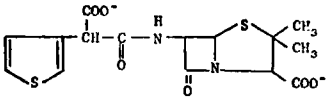

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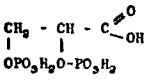

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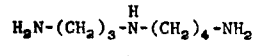
Miscellaneous Compounds - Suloctidil[49] (11), an anti-spastic (vasoactive) agent was reported to inhibit phospholipid and collagen-induced aggregation. This compound is similar to dipyridamole but more potent. Halofenate (12), a hypolipidemic[50] and uricosuric[51] agent, has been reported to inhibit platelet aggregation.[52] Several antibiotics of the penicillin-carbenicillin type have previously been reported to affect coagulation and/or platelet function.[53] Ticarcillin (13) was reported to affect platelet aggregation in human patients given therapeutic levels of the antibiotic.[54]

2,3-Diphosphoglycerate (2,3 DPG, 14), a naturally occurring material that increases in people with anemia, was reported to inhibit the second phase of platelet aggregation.[55] The inhibitory effect was potentiated by dipyridamole and Vinca minor alkaloids.

Rennert et al.[56] reported that naturally occurring polyamines spermidine (15) and spermine (16) inhibited ADP-induced, but not epinephrine-induced platelet aggregation. This inhibition was related to increases in these polyamines in leukemia, psoriasis, etc., and to increases in bleeding times. Synthetic diamines and polyamines have been reported previously to affect platelet aggregation.[57]



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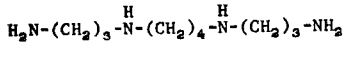

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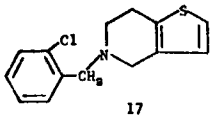

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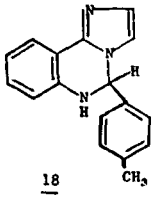
Ticlopidine (17) was reported to inhibit ADP-induced platelet aggregation when administered orally to 6 human volunteers. Doses of 250-1000 mg/day for 1 week gave inhibitions of 40-75%.[58]

The effect of derivatives of imidazo[1,2-c]quinazoline on platelet aggregation was reported.[59] Many showed activity, which was not limited to 5,6-dihydroimidazo[1,2-c]quinazolines. The most active was a 2,3-dihydro derivative (18). These compounds are related to BL-3459, which was previously reviewed.[15,60,61]

The inhibitory effect of nitrofurantoin (19) and several analogs on primary phase of ADP-induced platelet aggregation has been reported.[62] The authors suggest that the nitro group on the furan ring and the specific arrangement of the two keto groups on the imidazole ring are required for activity.

Several lactamimides have previously been reported to have inhibitory activity on platelet aggregation induced by ADP, epinephrine, collagen, thrombin, and serotonin.[13,63,64,65] A new series of lactamimides was recently reported to also inhibit platelet aggregation in vitro by the same inducers.[66] One of the series, RMI 12,366A (20), inhibited ADP-induced platelet aggregation when given orally to guinea pigs.



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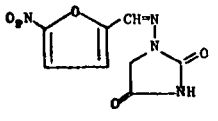

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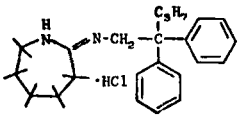

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Anticoagulants

In the formation of the fibrin clot, there are numerous steps involving many different procoagulant factors. Both intrinsic and extrinsic systems, along with platelet function and blood flow patterns, play a role in the formation of the fibrin clot. At the point of convergence of both intrinsic and extrinsic coagulation systems, the final product is an enzyme referred to as Factor X_a, or Autoprothrombin C. This enzyme along with cofactors (Factor V, phospholipoprotein - Platelet Factor 3, and calcium ion) catalyzes the formation of the enzyme thrombin from prothrombin. Thrombin in the presence of calcium ion produces the fibrin clot from fibrinogen. Both Factor X_a and thrombin are serine containing enzymes classified as serine esterases and proteases. They have a large part of their peptide chains in common and have been considered as part of the same precursor prothrombin.[67]

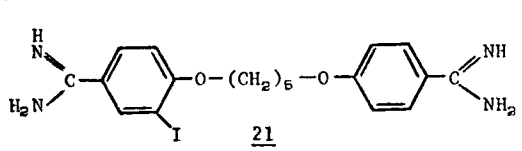
In the last several years, it has become known and more appreciated that the in vivo role of heparin as an anticoagulant may be less important as an inhibitor of thrombin and more important as an inhibitor of Factor X_a.[68] Actually heparin is not an anticoagulant, but a cofactor for a protein (α_2 globulin - antithrombin III) in the plasma that neutralizes Factor X_a or thrombin by molecular combination. Without heparin this neutralization, which is concentration dependent, is slow. In the presence of heparin, it is greatly accelerated. Heparin has no effect on thrombin

activity in a purified Fibrinogen-Thrombin-Ca^{++} system.

Mini-dose or subcutaneous heparin was reported on briefly in previous reviews.[13,14] In 1975-76 more clinical evaluations have been reported.[69-71] These smaller doses with lower and more sustained effect are efficacious in vivo for use before surgery and other thrombotic conditions to prevent venous thrombosis and pulmonary embolism. This new form of dosing was advanced by the development of new understanding of mechanism and development of new methods for measuring activity. Such therapy eliminates the necessity of laboratory monitoring and the danger of bleeding.

A better understanding of the mechanism of action of anti-vitamin K compounds has been gained. They lower blood levels of an active prothrombin molecule by prevention of CO_2 uptake on the glutamic acid residues in the molecule that allows Ca^{++} binding.[72] These compounds are discussed in a review by O'Reilly.[73] No new compounds of this type are reported.

Several new compounds have been reported that affect the formation of a fibrin clot. Aromatic diamidines, such as 21, were reported to inhibit several proteolytic enzymes including thrombin.[74] Concanavalin A (a globulin protein from the jack bean) inhibits fibrin formation by inhibiting the lipoprotein cofactor in the production of thrombin and thus decreasing the rate of thrombin production.[75] Several antibiotics (penicillins and cephalosporins) have been reported to affect fibrin clot formation as well as platelet function. Cephalothin (22) has been shown to delay fibrin polymerization and thus prolong the activated partial thromboplastin time (APTT) and thrombin time tests.[76]



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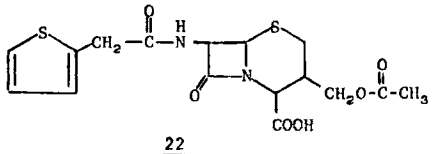

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The phospholipase A_2 from Vipera Berus venom was purified and its properties were determined[77] (mol. wt. 13,400 and isoelectric point 9.2). This enzyme hydrolyses the acyl group at the 2 position of phospholipid, thereby reducing the procoagulant activity of the phospholipoprotein cofactor in thrombin production.

A very simple compound, cyanate, was found to inhibit coagulation when used for treatment of blood from sickle cell anemia patients.[78] It decreased the levels of Factor V and X activity (19 and 36% respectively), inhibited Factors VII, IX, and XI (63-75%), and inhibited thrombin 80%.

Fibrinolytic Agents

Fibrinolysis has previously been reviewed.[79,80] The physiologic process in which a fibrin clot is dissolved and the regulation of that process is not completely understood. The main proenzyme in the blood is plasminogen, a β_2 globulin, which when activated is converted to the fibrin lysis enzyme plasmin, a β_1 globulin. Plasmin is usually bound by many other proteins and inactivated in the plasma. If this did not occur, pro-

teolysis of blood proteins and exposed cells would be a continual occurrance. Physiologically the plasminogen is trapped within the clot and an activator is required to permeate the clot to activate the lysis enzyme. Activators are always present in blood at very low concentrations. One of the important activators comes from the endothelial cells of the blood vessel wall. The ability to control the level of activators or reduce any abnormal inhibition of lysis is the aim of research toward fibrinolytic agents.

Since lipid (lipoprotein) inhibit lysis, agents that lower lipid levels have a normalizing effect on decreased fibrinolysis activity due to elevated lipids. Even the removal of normal lipid content from plasma with chloroform increases lysis activity. Vasoactive agents have previously been reported[81] to stimulate activator release resulting in enhanced lysis activity of short duration.

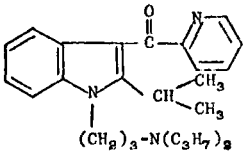
The normal tissue lysis activators are proteins. One such activator from the kidney is urokinase. This enzyme has not been found systemically, but only in the kidney and urine. It directly activates plasminogen to plasmin by hydrolysis of a small peptide from the plasminogen molecule. Other known enzymatic activators are streptokinase and brinase, which have been discussed elsewhere.[82,83] Urokinase can be isolated from urine in small amounts. This has made it a very expensive material. Abbott Laboratories has developed a tissue culture technique in which kidney cells are grown under exacting conditions and urokinase is isolated.[84] Recently Abbott Laboratories made a request to the FDA for a license to sell this form of urokinase called Abbokinase®. This process should increase the availability of urokinase and decrease its cost.

Another agent recently reported is hementerin isolated from the Brazilian blood sucking leech Haemerteria lutzi.[85] The activity of this material is similar to that of streptokinase, i.e. it is active in the human clot system, but not in the bovine system.

There are many synthetic chemicals that have been reported to increase lysis activity, chloroform being one of the earliest reported.[86] More recently acid anti-inflammatory,[87] antidiabetogenic,[88] vasoactive,[81] and diuretic agents,[89] and anabolic steroids[90] have been reported to have some effect on the lysis system. Even whiskey (alcohol) has been reported to enhance lysis.[91] Hedner, et al.[92] reported that ethyloestrenol (8 mg/day) increased spontaneous lysis in 45 patients with deep venous thrombosis and pulmonary embolism after 3 months of treatment. Euphillin (aminophylline) was reported to increase abnormally low fibrinolysis in human patients and in rabbits with venous thrombosis.[93] When 40 mg of furosemide was injected i.v. into 33 healthy people, the euglobulin lysis time was shortened.[94] Since this activation was not found in patients with uremia and nephrectomy, an intact renal system appears necessary.

Many synthetic anti-inflammatory compounds have previously been reported to have "fibrinolytic activity." The exact mechanism of action of these compounds (mostly in vitro), is not known. Some have been shown to inhibit inhibitors present in plasma or serum.[95] Usually these compounds

produce biphasic responses. Types of compounds recently cited include substituted indoles, such as 23,[96] 2-phenethynylcyclopropanecarboxylates, such as 24,[97] branched benzoic acids, such as 25,[98] and two series[99] related to the bis(tetrahydroisoquinoline)bisobrin,[15] the bis(benzylamine) (26), and a combination of these two structures, such as 27.



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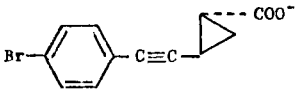

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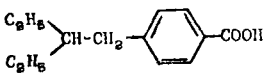

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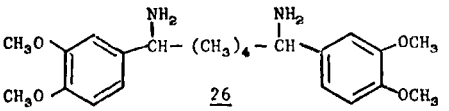

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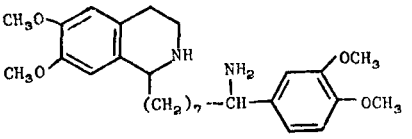

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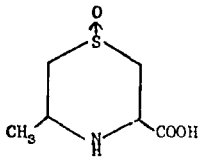

28

It has been reported[100] that ingestion of onions enhances fibrinolysis. In an attempt to isolate and characterize the active components, one active component has been identified as cyclo-alliin (28).[101] At 125 mg/subject, it enhanced lysis by 42% in the euglobulin lysis time test. Steam distilled and ether extracted onions enhanced activity 50-87%. Other sulfur containing oils were also discussed. Since they did not have an effect on the in vitro hanging clot method but were active in vivo, the authors suggested that either these substances weaken blood clots by opening disulfide cross links between fibrin molecules through a disulfide exchange reaction and therefore render them more susceptible to natural fibrinolysis, or they may stimulate liberation of activator.

Summary - Many diverse novel compounds that inhibit different platelet functions show great promise, not only for potential anti-thrombotic agents, but also for more specific effects on prostaglandin and/or thromboxane A_2 synthesis, and serotonin or calcium uptake and release. Many active compounds can be used as tools in the search toward a more complete understanding of the physiologic interactions of the hemostatic mechanisms. This better understanding would lead to the development and use of more potent and selective synthetic compounds in the inhibition of platelet aggregation and fibrin formation, and in the enhancement of fibrinolysis for the control of both arterial and venous thrombosis. It is hoped that some of these new compounds will be evaluated clinically in the near future.

Acknowledgement: I wish to thank the library staff of Merrell-National Laboratories, especially Mrs. Emily Rahe, for their help in the literature search for this review.

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Chapter 10. Agents Affecting Gastrointestinal Functions

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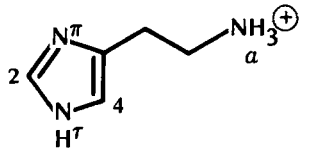
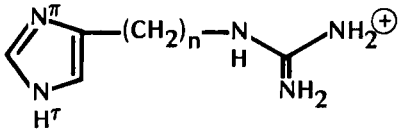
Introduction – Gastrointestinal drug research continues to highlight the development of histamine (H_2) receptor antagonists for use in treating peptic ulcer disease and other secretory related disorders. The introduction of Tagamet (cimetidine), the first clinically acceptable histamine-H_2 blocker, culminates the search for a safe and effective gastric acid antisecretory drug. Prostaglandin antisecretory drug development continues to be hindered by the presence of side effects. However, commercial interest in this class of agents continues and improved target selectivity could lead to rapid clinical advances. Developments in understanding histamine receptors and the pharmacology of histamine antagonists along with progress in prostaglandin research are the focus of this review. The gastric acid antisecretory structure-activity relationships and clinical testing of gastrointestinal peptide hormones are also updated with a view toward avoiding overlap with other excellent summaries.

Detailed reviews of the gastrointestinal actions of prostaglandins[1,2,3,4,5] and gut hormones[6,7] have been published recently along with the proceedings of a second international symposium on H_2 antagonists.[8] An excellent overview of the pharmacological and clinical profile of carbenoxolone was published[9] recently and will not be treated in this chapter.

Histamine Agonist activity – Theoretical molecular orbital calculations[10] along with X-ray data[11] indicate that histamine monocation exists as the N^{τ}-H tautomer while histamine free base exists as the N^{π}-H tautomer. Deprotonation of the ethylamine side chain nitrogen is electronically equivalent to interaction of the charged side chain nitrogen with an anionic binding site and leads to a proton shift from the N^{τ}-H to the N^{π}-H tautomeric form of the imidazole ring.[12] In a model proposed for H_2-agonist activity,[12,13] histamine N^{τ}-H tautomer binds to a receptor site via hydrogen bond donor and acceptor bonding. Interaction of the charged ethylamine side chain nitrogen with an anionic site triggers the tautomer change and leads to H_2 agonist activity. Removal of the tautomeric H by methylation of either the N^{τ}-H or N^{π}-H histamine tautomer[14] or replacement of the imidazole ring by nontautomeric ring systems such as pyridine or thiazole[13] leads to loss of H_2 agonist activity. Conversion of the histamine side chain N^{α} amino group into a guanidine moiety leads to a molecule having the correct N^{τ}-H imidazole ring tautomer for receptor binding but having a side chain moiety markedly different in basicity and geometry from that of histamine. This compound and its homo side chain analog[16] are partial agonists at the histamine H_2-receptor and at high doses inhibit histamine-stimulated acid secretion. Methylation of the histamine imidazole ring at C_4 or bismethylation at C_4 and N^{α} leads to compounds that exist predominantly as N^{τ}-H tautomers at physiological pH and have selective histamine H_2-agonist activity.[14]



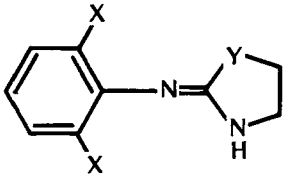
histamine monocation



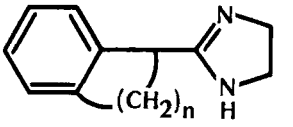
n = 2, 3 partial H_2 agonists

Tolazoline and the structurally related imidazoline tetrahydrozoline may be H_2 receptor agonists since tolazoline-induced acid stimulation in the dog can be blocked by burimamide, metiamide and cimetidine[17] and both imidazolines induce characteristic H_2-agonist effects on isolated guinea pig atria preparations which can be blocked by metiamide.[18] Clonidine, like the imidazolines, has α-adrenergic agonist activity

along with H_2 agonist activity[19] and stimulates acid secretion in anesthetized rats. However in conscious rats clonidine and other structurally related imidazolines and pyrrolidines possess gastric acid antisecretory and antihypertensive activity. The antisecretory effect of clonidine in rats may be explained by a centrally mediated H_2-agonist effect[20] which results in acid inhibition. In anesthetized animals the central effect is masked and only acid stimulation caused by a peripheral H_2 agonist effect is observed. Among a series of clonidine analogs 2-(2,6-Dimethylphenylimino)imidazoline (I) and 2-(2,6-dichlorophenylimino)pyrrolidine (II) are particularly effective antisecretory agents in conscious rats with minimal antihypertensive activity.[21]

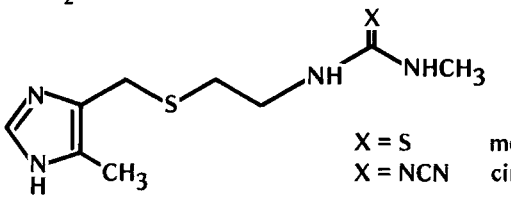


X = Cl, Y = NH Clonidine
X = CH_3, Y = NH (I)
X = Cl, Y = CH_2 (II)



n = 0 Tolazoline
n = 3 Tetrahydrozoline

Pharmacology of Cimetidine – Cimetidine differs from its precursor metiamide by having a cyanoguanidine replace the thiourea moiety found in metiamide. Reversal of metiamide-induced agranulocytosis in patients by instituting cimetidine therapy[22] suggests that this toxic effect is due to the thiourea functionality and is not a characteristic of H_2 antagonists. Following the observation of agranulocytosis in two patients



X = S metiamide
X = NCN cimetidine

receiving metiamide, cimetidine advanced to human clinical pharmacology and ulcer healing trials. The animal gastrointestinal pharmacology for cimetidine has been reviewed[23] and apart from demonstrations of ulcer healing studies in rats[24] few additional animal findings are reported. Inhibition of acid output is the key action for H_2-antagonists and in man cimetidine decreases meal-stimulated acid secretion in both healthy volunteers[25,26,27] and duodenal ulcer patients.[28,29,30] Histamine-,[27] methyl xanthine-[31] (caffeine) and vagal-[32] (insulin) stimulated acid output are also blocked. Postprandial serum gastrin concentrations following cimetidine are not significantly different from placebo controls when intragastric pH is maintained constant but are elevated if gastric pH fluctuates naturally.[29] Inhibition of acid output (IC_{50}) correlates with peak serum cimetidine concentrations of approximately 1.6-2.0 μmoles/l when given intravenously.[26,27] Peak blood levels of cimetidine occur 45-75 min following oral doses and decrease with a half-time of approximately 124 min.[23,27] If drug is taken with a meal absorption is delayed and peak levels occur when the buffering capacity of the meal is decreasing.[33] Cimetidine is excreted primarily in the urine as unchanged drug or the sulphoxide metabolite following either oral or intravenous administration and is largely cleared within 24 hrs.[27] Gastric acidity in both normal volunteers[25] and duodenal ulcer patients[30] is controlled with 0.8-1.0 g/day in studies designed to mimic normal levels of activity. In one study decreased acidity correlated with an approximate 33% increase in electrical potential difference suggesting that cimetidine enhances the mucosal barrier in addition to reducing acid output.[34] The interpretation of the study has been questioned, however.[35,36] Cimetidine at antisecretory doses does not interfere with other gastrointestinal functions including intrinsic factor secretion,[37] lower esophageal sphincter function,[38] gastric emptying and pancreatic or bile acid output.[39] Because the tone of the

esophageal sphincter is maintained at dose levels known to inhibit acid output, cimetidine may be useful in patients with chronic gastroesophageal reflux.

Agranulocytosis occurred during clinical use of metiamide but is not reported for cimetidine. Concern for possible immunological consequences of prolonged H_2 antagonist administration has been expressed since histamine modulates cellular immune and inflammatory functions.[40] In patients given cimetidine for up to 8 weeks, however, no significant changes occur in leucocyte migration inhibition, autoantibody production or serum immunoglobin concentration.[41]

Use in Duodenal Ulcer Disease – Clinical trials in duodenal and prepyloric ulcer patients have established 6-week healing rates of 80-90% in cimetidine treated patients compared with 25-42% in placebo groups.[42,43,44] Comparative ulcer healing trials with traditionally prescribed anticholinergics have not been reported; anticholinergic drugs do not, however, potentiate the antisecretory effects of cimetidine obviating any recommendations for concurrent use with the H_2-antagonist. The significant increase in ulcer healing incidence reported for cimetidine treated patients correlates well with symptomatic improvement and decreased antacid consumption. Cimetidine efficacy for treating duodenal ulcer disease appears unrelated to the patients' prior duration of illness, level of gastric acid secretion or recurrence record.[45] The effect of cimetidine on prevention of ulcer relapse is unclear but in one study an early relapse rate of 41% occurred suggesting that some patients may require intermittent or chronic therapy for lasting control of the disease.[46] The basis for the high relapse rate is unclear, however, since preliminary findings in 19 DU patients treated for 3 mos. with cimetidine (1.6 g/day) suggest that continuous treatment reduces parietal cell mass.[47]

Use in Gastric Ulcer Patients – In contrast to duodenal ulcer disease, efficacy for the use of cimetidine in gastric ulcer disease is not clearly established. In an uncontrolled G.U. study, complete healing occurred following 6 weeks of cimetidine treatment in ten patients.[48] Symptomatic relief and reduction of antacid consumption accompanied healing but acute atrophic gastritis remained. Some corroboration of this experience has been reported.[49] Historically, however, gastric ulcer patients have high spontaneous healing rates and greater clinical exposure is necessary to establish efficacy for gastric ulcer disease.

Zollinger-Ellison Syndrome – Cimetidine also blocks acid output in Z-E patients without causing side effects.[22,50] This use of an H_2-receptor antagonist, shown earlier for metiamide,[51,52] represents an important advance in the treatment of this peptic ulcer disease condition.

Peptides-Gastrin – Structure-activity studies on peptide analogs of the C-terminal tetrapeptide amide of gastrin (tetragastrin) indicate that a variety of structural changes lead to acid stimulatory or inhibitory compounds of low potency. Replacement of the C-terminal amide by a methyl ester or oxidation of the sulfur atom of the methionyl residue to a sulphone moiety decreases acid stimulatory activity in dogs to one-twenty-fifth of the tetrapeptide amide.[53] Replacement of aspartic acid by glycine in tetragastrin or by tyrosine O-benzyl ether in an N-terminal benzyloxy carbonyl derivative of tetragastrin yields compounds with very weak acid antisecretory activity in rats.[54]

Gastrin – Gastrin may possibly be important in other disease states. Elevation of serum gastrin levels have been reported in rheumatoid arthritis patients[55] and elevated gastrin levels have been hypothesized to contribute towards maturity onset diabetes.[56]

Gastrin Release – Factors that influence gastrin release are particularly important because gastrin is a potent stimulant of both acid secretion and mucosal cell growth. It is clear, however, from the summary shown in Table 1 that the pharmacology of gastrin release is poorly understood.

Table 1. Factors that Affect Gastrin Release In Vivo

Species	*Drug/Agent*	*Effect on Serum Gastrin Concentration*	*Ref.*
Cat	Atropine	Decrease	57
Dog	Atropine	No effect	58,59
	Bombesin	Increase	60
	Prostaglandin E_1	Increase	61
Man	Aminophylline	Increase	62
	Arginine	Increase	63
	Atropine	Increase	64
	Bombesin	Increase	65
	Calcium	Increase	66,67
	Insulin	Increase	68
	Isoproterenol	Increase (D.U. patients)	69
	Streptozocin	Increase	70
	Prednisolone, ACTH	Increase	71
	Cimetidine	No effect, increase	29
	Indomethacin	No effect	72
	Propranolol	No effect, decrease	73,74
	Secretin	Decrease	75
	Somatostatin	Decrease	76,77
	Sulpiride	Decrease	78
	16,16-dimethyl PGE_2	Decrease	79
	Glucagon	Decrease	80
	Calcitonin	Decrease	81
Pig	Parathyroid Hormone	Increase	82

In man, several peptide hormones lower gastrin levels while β-adrenergic stimulation increases serum gastrin levels. Sulpiride, an ulcer healing agent, lowers gastrin levels possibly by an action on the central nervous system. 16,16 Dimethyl PGE_2 reduces gastrin concentrations concomitantly with gastric acid antisecretory effects only when gastric pH is maintained constant (intragastric titration). Drug effect on gastrin levels is unknown when pH is allowed to fluctuate naturally. A procedure to obtain enriched numbers of isolated gastrin cells has been reported and may allow detailed *in vitro* pharmacological studies of gastrin release to proceed.[83]

Trophic Action of Gastrin – The physiological role of gastrin as a trophic hormone is established and has been reviewed.[84] The trophic action is independent of the stimulation of acid secretion and is not affected by dose levels of H_2-antagonists[85] or prostaglandins[86] known to block acid output. Cholecystokinin-octapeptide (CCK-OP) has a weak trophic effect on the duodenum but not the stomach and in rats CCK-OP acts as a weak competitive inhibitor of gastrin's trophic action. In animals, growth hormone appears necessary for the maintenance of normal serum and antral gastrin levels suggesting that gastrointestinal function is dependent on a pituitary-antral axis.[87] In man, pentagastrin but not synthetic human gastrin stimulates the rate of cell proliferation in the normal fundic mucosa.[88] Clearly, it is necessary to extend the knowledge of gastrin's trophic function to man since prostaglandin and H_2-antagonist antisecretory agents are likely to elevate serum gastrin concentrations in peptic ulcer patients during periods of treatment.

Gastric Inhibitory Polypeptide – (G.I.P.) is a 43 amino acid polypeptide released by glucose and fat ingestion. In addition to its insulinotrophic effects in animals and man, G.I.P. inhibits acid secretion in

dogs. Fat ingestion in man elevates serum immunoreactive G.I.P. levels[89] suggesting that this peptide, at least in part, may be involved in the known inhibition of acid secretion by fat in the duodenum. The total synthesis of porcine G.I.P. has now been reported. Interestingly, the N-terminal 28 amino acid sequence, which structurally corresponds most closely to the 28 and 29 amino acid sequences of the gastric acid antisecretory peptides, vasoactive intestinal peptide (V.I.P.) and secretin, does not have antisecretory activity.[90] The C-terminal octadecapeptide of G.I.P. (amino acid residues 26 to 43) is devoid of antisecretory activity in histamine-stimulated dogs[91] but peptide chain extension to the C-terminal nonacosapeptide (residues 15 to 43) results in material having one fourth the antisecretory potency of G.I.P. in tetragastrin-stimulated dogs.[92] Totally synthetic G.I.P. inhibits both histamine- and tetragastrin-stimulated acid secretion in dogs and is about 4 times more active than G.I.P. isolated from natural sources.

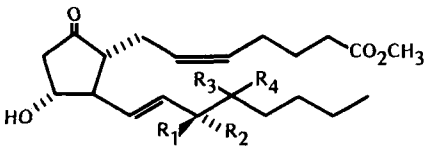
Secretin – inhibits acid secretion, stimulates pancreatic bicarbonate output and lowers serum gastrin levels. Since these properties would benefit duodenal ulcer (D.U.) patients secretin has been recently tested in 2 short clinical trials. In a 10 day trial 7 D.U. patients receiving secretin subcutaneously every 4 hours achieved significant gastric alkalinization but no effect on pain relief or duodenal ulcer healing was observed.[93] In a 3 week trial complete pain relief was observed in 8 D.U. patients using a depot secretin preparation; 5 of 8 patients were ulcer-free two weeks after treatment was started.[94] The significance of secretin therapy on the duration of duodenal ulcer healing is still uncertain and rapid progress appears unlikely in view of the high cost and low availability of synthetic secretin.

Somatostatin (growth hormone release inhibiting hormone) – is synthesized by endocrine-peptide producing cells of the APUD series and, in common with an increasing list of hormonal peptides, is localized in both CNS and gastrointestinal tissues. The common embryonic origin of these tissues has been recently reviewed.[95] In addition to being localized in the hypothalamus and pancreas somatostatin is widely distributed throughout the upper gastrointestinal tract of rat,[96] dog, pig and man.[97] The close proximity of somatostatin-containing D cells to gastrin-containing G cells in rat pyloric antrum[98] suggests that somatostatin has a local role in the regulation of gastrin release. Given systemically somatostatin inhibits basal and food-stimulated gastrin release in animals[99,100] and man[101] and, in contrast to secretin, inhibits gastrin release from pancreatic tumors of Zollinger-Ellison syndrome patients.[76,102] Somatostatin's marked gastric acid antisecretory activity in food-stimulated animals and man may be partly due to depression of serum gastrin levels. However, somatostatin also exerts a direct antisecretory effect on the parietal cell since it inhibits pentagastrin-stimulated acid secretion.[103] Although prolonged somatostatin infusion has been used to control bleeding and speed healing in an ulcer patient[104] the utility of this agent in ulcer therapy is limited by the short half-life, and lack of tissue specificity and oral activity. Synthetic modifications which prevent metabolism by aminopeptidases, and carboxypeptidases or which prevent disulfide bridge reduction produce analogs with gastric acid antisecretory activity comparable to somatostatin but with prolonged duration of action.[105] Replacement of L-Lysine[9] by D-Lysine[9] decreases acid inhibitory activity.[106]

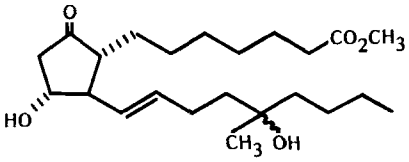
β and γ Urogastrone – are two structurally related 52 and 53 amino acid containing peptides which differ in the possession of a C-terminal arginine residue.[107] The urogastrones which are isolated from normal male urine, are structurally related to mouse and possibly human epidermal growth factor (EGF).[108] Both urogastrones[109] and EGF[110] are potent inhibitors of acid secretion in animals and man and at somewhat higher doses stimulate epithelial growth. Since the molar potency of urogastrone as an acid inhibitor slightly exceeds that of gastrin as an acid stimulant sufficient material has been obtained for clinical trials despite yields of 1 μg peptide per liter urine. In Zollinger-Ellison syndrome patients urogastrone reduces acid secretion by 50 to 80%[111] but in contrast to studies in normal volunteers[109] significantly elevates plasma-gastrin levels. Patients had complete pain relief which persisted for up to 24 hrs following urogastrone infusion. Some simplification of urogastrone structure is compatible with good antisecretory activity since the penultimate C-terminal pentapeptide is not necessary for activity.[108] Total synthesis of the urogastrones which contain three disulfide bonds has not yet been reported.

Prostaglandins Metabolic Stability – Research on gastrointestinal uses of prostaglandins continues to focus on analogs with enhanced oral activity and greater tissue selectivity. Methyl esterification of the carboxyl group of PGE_1 increases intravenous gastric antisecretory potency in dogs probably by retarding metabolic β-oxidation of the carboxylic acid side chain.[112] In animals the methyl ester of 16S methyl-13-dehydro PGE_2 exhibits oral gastric antisecretory activity while the parent acid is only weakly active. Given intravenously the parent acid is a more potent antisecretory agent than PGE_2 with fewer side effects.[113] Prolonged antisecretory action of 15 and 16-methyl substituted synthetic analogs is due to resistance to degradation by prostaglandin 15-hydroxydehydrogenase.

Structure-Activity Studies – Homologation of the terminal alkyl side chain in PGE_1 results in analogs with enhanced tissue selectivity. In particular, ω-Ethyl PGE_1 has one fourth the antisecretory potency of PGE_1 in dogs but only one fifteenth of the effect of PGE_1 on smooth muscle. Moving the 15-hydroxyl group to carbon-16 in a derivative of PGE_1 methyl ester (SC-28,904) improves antisecretory activity in dogs and decreases smooth muscle stimulating activity. Further displacement to the 17 position causes almost complete loss of activity in both areas.[115,116] Simplified prostaglandin analogs lacking the hydroxylic bottom side chain show very weak antisecretory effects.[117]



R_1	R_2	R_3	R_4	
CH_3	OH	H	H	(15S)15-CH_3 PGE_2 methyl ester
OH	CH_3	H	H	(15R)15-CH_3 PGE_2 methyl ester
H	OH	CH_3	CH_3	16,16 di-CH_3 PGE_2 methyl ester
H	OH	H	H	PGE_2 methyl ester



SC 29,333

Animal Pharmacology 15 Methyl PGE_2, 16,16 Dimethyl PGE_2 – Given intravenously, 15-methyl and 16,16 dimethyl PGE_2 are respectively 40 and 100 times more potent as acid antisecretory agents in dogs than PGE_2.[118] Although pepsin levels are not reduced concomitantly with acid output, protection from induced ulceration can be observed and both compounds are active when given orally. In dogs, 16,16 dimethyl PGE_2 inhibits histamine-stimulated acid secretion most effectively followed by urecholine, pentagastrin and 2-deoxy-D-glucose.[119] In rats (15S)-15 methyl and 16,16 dimethyl PGE_2 are 40 times more active than PGE_2 at reducing pentagastrin-stimulated acid output but also increase intestinal motility *in vivo* suggesting only modest improvements in selectivity of action.[120] (15S)-15 methyl PGE_2 is also a more potent inhibitor of acid output in monkeys than PGE_2 by a factor of at least 10, intravenously, or 300 intragastrically using histamine as the secretagogue.[121] In contrast to dogs, monkeys appear to be less sensitive to the side effects (emesis, diarrhea) of parenterally administered prostaglandins and may be better experimental test subjects for predicting side effects in man.[122] Concern for the side effects, particularly diarrhea, common to prostaglandin analogs is shown by the development of an assay to test for diarrheagenic actions.[123] Absence of significant side effects is particularly important since the H_2 receptor antagonist, cimetidine, is relatively tissue selective.

Animal Pharmacology SC-29,333 – Some improvement in antisecretory potency and selectivity is reported for a methylated PGE_1 analogue (SC-29,333) in which the C-15 hydroxy moiety has been transposed to the adjacent C-16 position. Using PGE_1 methyl ester as a reference standard, SC-29,333 is approximately 30 times more potent, intravenously. Given orally, SC-29,333 is also a longer acting antisecretory drug than PGE_1 methyl ester and appears somewhat better tolerated.[124]

Mechanism of Prostaglandin Action – The cellular mechanism of prostaglandin inhibition of acid secretion is unclear but some evidence suggests that prostaglandins increase cyclic AMP concentrations by stimulating adenyl cyclase[125] and enhance active sodium transport[126] in the gastric mucosa. Interestingly, instillation of 16,16 dimethyl PGE_2 into dog gastric pouches causes moderate disruption of the gastric mucosal barrier resulting in back diffusion of hydrogen ions and net sodium movement into the pouch.[127] In contrast, metiamide, an H_2 receptor antagonist does not change ionic permeability. The significance of the finding is unclear. Ulcer healing activity of prostaglandins may not be due entirely to antisecretory activity since indomethacin-induced intestinal ulceration in rats is effectively blocked by $PGF_{2\alpha}$ along with other prostaglandins which have only weak gastric antisecretory activity.[128]

Human Studies 16,16 Dimethyl PGE_2 (DMPG) – In normal volunteers, DMPG inhibits basal,[129] pentagastrin-[130] and histamine-stimulated[129,131] gastric acid output; pepsin is unaffected. In duodenal ulcer patients, DMPG (0.75-1.77 μg/kg) significantly reduces acid secretion (61-94%) and serum gastrin concentrations following a meal.[77] When given intraduodenally, DMPG is a less effective inhibitor of gastric acid output and gastrin release. Results of one study suggest that duodenal ulcer patients are less susceptible to the gastric antisecretory action of DMPG than normal subjects.[132] DMPG like PGE_2 inhibits pancreatic bicarbonate secretion in dogs. In man, however, DMPG does not inhibit bicarbonate secretion although pancreatic enzyme output is elevated in both dogs and man.[133] In addition to a well characterized gastric acid antisecretory action DMPG significantly reduces motor activity of the gastric antrum and duodenum following high oral doses.[134] Enhanced gastric emptying and a dilation of the proximal duodenum suggest that decreased resistance to outflow may be responsible for the enhanced emptying observed with this analog.[135]

Human Studies 15 Methyl PGE_2 – (15R) Methyl PGE_2 methyl ester lacks intrinsic acid antisecretory activity. Acid antisecretory activity is observed following oral dosing in dogs and man or after preincubation with acid and is due to acid catalyzed isomerization of the allylic C-15 hydroxyl to a 50:50 mixture of (15R) and the active (15S) methyl isomer.[136] Large antisecretory doses of the (15S) methyl isomer cause abdominal discomfort and diarrhea. Variable side effects[137,138] following oral dosing with the (15R) isomer thus probably reflect the extent of isomerization to the (15S) epimer. In healthy volunteers intragastric doses of 1.5 μg/kg of 15(S) 15-methyl PGE_2 methyl ester completely inhibit meal-induced acid secretion.[138] In duodenal ulcer patients intravenous doses of 0.5 μg/kg/hr reduce meal-induced acid secretion by 40%.[139] Serum gastrin levels are reduced following intragastric but not intravenous dosing. Interpretation of drug effect on gastrin levels in these studies is difficult because of marked differences in dose, experimental protocol and type of subject studied. Oral doses of 100 to 200 μg 15(R) 15-methyl PGE_2 (free acid) inhibit basal acid secretion in man.[140] In addition to inhibitory effects on acid secretion and stimulatory effects on mucus production, 15(R) 15-methyl PGE_2 methyl ester also inhibits pepsin secretion in man. In a small double blind trial in gastric ulcer patients 15(R) 15 methyl PGE_2 (free acid) given orally in doses of 150 μg every 6 hrs for two weeks significantly increased the rate of ulcer healing using endoscopic assessment.[137] The very low rates of healing observed in the placebo group in this study may make comparisons with other gastric ulcer healing studies difficult since the secretory characteristics of Chinese gastric ulcer patients appear different from those of Caucasian gastric ulcer patients.[141] Ulcer healing studies in duodenal ulcer patients have not been reported. PGE_2 is also reported to promote gastric ulcer healing in a small double blind trial.[142]

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Section III - Chemotherapeutic Agents

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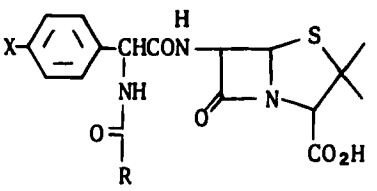
Chapter 11. β-Lactam Antibiotics

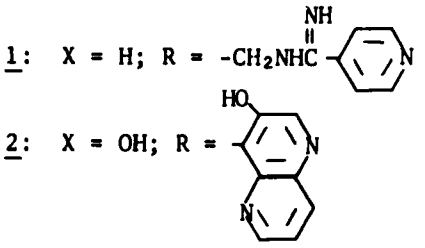
J. Alan Webber, Eli Lilly and Company, Indianapolis, Indiana

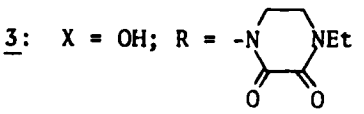
Penicillins - An interesting new penicillin derivative, RIT 2214, bearing a 6-(2-amino-2-carboxy)ethylthioacetamido side chain, was discovered in a fermentation broth.[1] RIT 2214 has broad-spectrum antibacterial activity, though less active than ampicillin, and was more active in vivo than ampicillin following subcutaneous administration to mice, possibly the result of higher blood levels.

Ticarcillin, 3-thienylmalonoyl 6-APA, has been closely compared to carbenicillin in vitro.[2,3,4] Although results vary, ticarcillin appears to be slightly more active against gram-negative pathogens, especially Pseudomonas aeruginosa. Clinical evaluation of ticarcillin suggests that it will be a useful drug in combating gram-negative infections,[5,6,7] possibly at a lower dose than carbenicillin. In vitro examination of mezlocillin and azlocillin has shown them to have some superiority to carbenicillin against a number of species of gram-negative bacteria,[8] including Bacteroides fragilis.[9] The clinical evaluation of these two new penicillins suggests adequate tolerance and effectiveness.[10] Mecillinam (FL 1060), the unusual amidino penicillanic acid derivative, has been shown to be synergistic with other β-lactam antibiotics (penicillins and cephalosporins) both in vitro[11] and in vivo.[12] Synergy with non-β-lactams is less pronounced.[12,13] Mecillinam itself has marked activity against the more sensitive species of gram-negative aerobic bacteria.[14] The majority of ampicillin-resistant Enterobacteriaceae strains isolated from patients were susceptible to mecillinam.[15]

Several new penicillins with expanded gram-negative spectra including activity against P. aeruginosa have recently been evaluated. Pirbenicillin (1) is more active than carbenicillin against P. aeruginosa and Klebsiella species, but less active against Proteus sp.[16,17] A study of pirbenicillin pharmacokinetics in mice concluded that it has a larger distribution volume than carbenicillin.[18] Compound PC 904 (2) has very good in vitro activity against most bacteria including Pseudomonas, Serratia, Klebsiella, and B. fragilis; its efficacy in mouse infections compared very favorably with that of carbenicillin.[19] Compound T-1220 (3) is also considerably more active than carbenicillin against a broad range of gram-negative bacteria.[20] It has low toxicity in animal tests and has proven effective in clinical trials.[21]





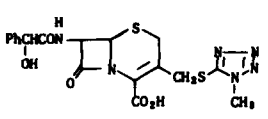


A class of penicillin derivatives in which the 3-carboxyl group is replaced by a tetrazole moiety is now known. The two analogues reported so far, CP-35,587[22] and CP-38,118,[23] suggest that the tetrazole compounds have a somewhat broadened spectrum of antibacterial activity. A large series of lactonyl esters of penicillins have been prepared and examined for hydrolysis rates and oral absorption. In general, the esters of ampicillin were orally absorbed in varying degrees,[24,25] while those of other penicillins were not.[25] The phthalidyl ester of ampicillin, talampicillin, looked best in experimental animals and was chosen for examination in humans. Talampicillin compared favorably with amoxycillin and gave higher blood levels than ampicillin in a comparative trial.[26]

Cephalosporins - Although cephalosporin C was the first naturally occurring cephalosporin derivative, and for many years remained the only one, recently several other derivatives of the cephem ring system have been isolated. A new compound in which the acetate of ceph C is replaced by a 1,1-dimethyl-2-amino-2-carboxyethylthio moiety was elaborated by a Cephalosporium acremonium mutant strain.[27] Also observed in a fermentation broth was 4-carboxybutanamido-7-ADCA, -7-ACA, and the latter's deacetyl analogue, all of which could have been accumulated through enzymatic action or de novo synthesis.[28] A new cephamycin (7-methoxy cephalosporin), C2801X, being the 3,4-dihydroxycinnamoyl analogue of cephamycin A and B, has been isolated.[29,30]

Interest continued in cephalosporins of proven or potential clinical relevance. Valuable perspective on the relative in vitro activity of clinically interesting cephalosporins has been provided.[31,32] The heaviest emphasis has been on candidates for parenteral administration. Cefazaflur (SKF 59962), for which human pharmacology had previously been reported, has excellent in vitro activity,[33,34] although its activity against more resistant gram-negative bacteria is not as great as that of other newer cephalosporins. Ceftezole (demethyl cefazolin) has comparable in vitro and mouse infection effectiveness to cefazolin.[35,36] Although lower in serum binding, ceftezole also shows a shorter half-life and lower peak serum level than cefazolin in humans.[36,37] Extensive animal

pharmacokinetic data have also been published.[37] A large clinical trial has demonstrated the effectiveness of ceftezole.[38] Cefamandole (4), cefoxitin (5), and cefuroxime (6) are cephalosporins having expanded gram-negative antibacterial spectra and are presently undergoing clinical trial. Cefamandole has been thoroughly examined *in vitro*,[39-42] studied for human pharmacokinetic parameters,[43-50] and shown to be clinically effective.[51] *In vitro* comparison studies including cefamandole and cefoxitin,[52-54] cefamandole and cefuroxime,[55] and cefoxitin and cefuroxime[56] have been reported. The results of a broad trial have also demonstrated the clinical effectiveness of cefoxitin.[57] Cefuroxime[58] has been examined for *in vitro*[59,60] and *in vivo*[61] characteristics. Favorable human pharmacokinetics[62] and clinical efficacy[63,64] have been established. The *in vitro* studies of these three cephalosporins have suggested some trends of relative activity against normally cephalosporin-resistant gram-negative bacteria. Cefamandole, cefoxitin, and cefuroxime are all active against a substantial percentage of indole-producing *Proteus* strains. Cefamandole and cefuroxime inhibit the growth of many *Enterobacter* strains, while cefoxitin has negligible activity. Cefamandole and cefuroxime are more active than cefoxitin against *Hemophilus influenzae* strains. Cefoxitin is somewhat more active against *S. marcescens* strains; and none of the three inhibits the growth of *Pseudomonas*.



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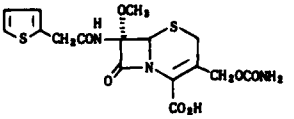

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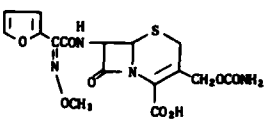

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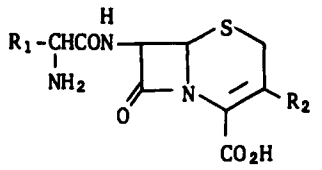
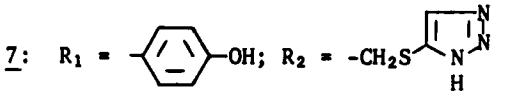
Fewer cephalosporins have been of clinical interest for oral administration. Studies of cefatrizine (BLS-640; SKF 60771) (7) have continued to determine in vitro characteristics,[65-67] human pharmacokinetics,[66,68,69] and clinical effectiveness.[69-71] Extensive clinical trial data documented the effectiveness of cefadroxil (BLS-578; p-OH cephalexin).[72] Cefaclor (Lilly 99638) (8) has been shown in *in vitro* studies to have better activity than cephalexin against gram-negative bacteria, especially *H. influenzae*.[56,60,73,74] The excellent oral absorption in mice, rats, and dogs paralleled that of cephalexin.[75] Metabolism was observed in dogs[76] but not in rodents. Human pharmacology studies indicated that cefaclor gave acceptable blood and urine levels.[77]

The evaluation of FR 10612 (m-methanesulfonylamino cephalexin) indicated that it was comparable to cephalexin *in vitro*, but more effective orally against experimental mouse infections, presumably due to enterohepatic recirculation also leading to higher and longer blood levels.[78] Although apparently dose related, human blood level curves for FR 10612 did show a longer half-life than did cephalexin. Oral cephalosporin candidate SCE-100 (tetrahydro cephalexin) has been examined extensively for *in vitro* and *in vivo* properties.[79] It is not as active as cephalexin.

CGP 9000 (9), a 3-methoxycephem derivative, is somewhat more active than cephalexin *in vitro* and is efficiently absorbed orally in mice.[80] The structure-activity relationship of a series of orally active cephalosporins



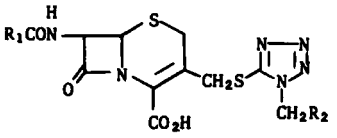


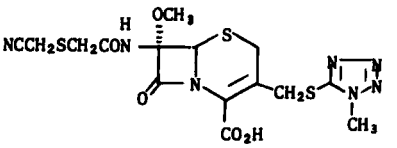
7: R_1 = ; R_2 =

8: R_1 = Ph; R_2 = -Cl

9: R_1 =  ; R_2 = $-OCH_3$

with phenyl- and hydroxyphenylglycyl side chains which led to the discovery of cefatrizine has been published.[81] An impressive array of 3-heterocyclicthiomethylcephem nuclei were included in the study. The superior influence upon oral absorption in mice of p-OH phenylglycyl over o-OH-, m-OH-, or phenyl- was demonstrated. The methyltetrazolethio and 1,2,3-triazolethio heterocyclic functions maximized *in vitro* activity. BLS-786 (10), a new cephalosporin with two hitherto unpublished side chains (o-aminomethylphenylacetyl and carboxymethyltetrazolethio), has *in vitro* activity comparable to cefamandole, with higher and longer blood levels similar to cefazolin.[82,83] It has excellent effectiveness in mouse therapy studies. An extensive study was reported of structure-activity relationships among cephalosporins containing certain substituted heterocyclicthio groups, with emphasis on substituted methyltetrazolethio.[84] Cefamandole analogues were stressed. The *in vitro* and *in vivo* activities were maximized in SKF 75073 (11).[85] This analogue was comparable to cefamandole *in vitro* (with, however, extraordinary *Proteus mirabilis* activity), had very high blood levels of long duration in mice, dogs, and squirrel monkeys, and cured experimental mouse infections very well. A series of 7-sulfonylacetamidocephems, which could be viewed as cefazaflur analogues, were synthesized.[86] Although some were quite active, none matched cefazaflur. Two cephalosporins with α-ureidophenylacetyl side chains and having modest *Pseudomonas* activity were reported.[87] Further refinements in the structure-activity relationships of α-sulfo cephalosporins have been published.[88] Included were variations in the aryl (α-sulfo) acetyl moiety and a number of 3-heterocyclicthiomethylene functions. The latter do not match the excellent *Pseudomonas* activity of the pyridinium-methyl compounds. The data prompted the authors to suggest that it is the combination of an acidic 7-side chain together with a positive charge attached to the 3-methylene position that leads to increased *Pseudomonas* activity while reducing inhibition of other gram-negative bacteria. Synthesis of the 3-cyanocephem system and others in which the C-3 carbon is included in a heterocyclic ring have been accomplished.[89] Although the gram-positive activity in this series approached that of cephalothin, the gram-negative activity was much lower.





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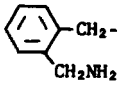
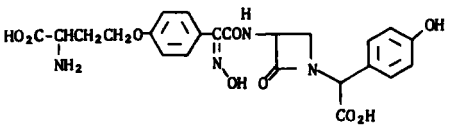
10: R_1 =  , R_2 = CO_2H

11: R_1 = $PhCH(OH)-$, R_2 = SO_3H

Interest has continued in 7-methoxycephems. An improved 7-methoxylation route has been developed,[90] as well as modification of an earlier route leading to incorporation of substituents other than methoxy.[91] The synthesis and evaluation of 7-methoxycephems related to cefazaflur were published.[92] The new compounds displayed the same general trend seen in other 7-methoxy derivatives, i.e., less gram-positive activity, less activity against cephalosporin-sensitive gram-negative bacteria, and enhanced inhibition of resistant gram-negative strains compared to non-methoxy analogues. A series of 7-methoxy-7-(2-substitutedthio)acetamidocephem derivatives were prepared and examined for in vitro activity.[93] The best compound was slightly more active than cefoxitin. A new 7-methoxycephem derivative, CS-1170 (12), has been prepared and found to be more active than cefoxitin.[94] Extensive data have been presented on the structure-activity relationships leading to this compound,[95] its in vitro and in vivo activity,[96] and the absorption, distribution, excretion, and metabolism in several species.[97]

Naturally Occurring β-Lactam Containing Compounds - Details of the discovery, isolation, and characterization of the unusual monocyclic β-lactam compound nocardicin A (13), previously known as FR 1923, have been published.[98,99] The novel bicyclic ring-oxygen containing compound clavulanic acid (BRL 14151) (14) has been isolated from fermentation of a streptomycete.[100] Clavulanic acid, although having a low order of antibacterial activity, inhibits the action of certain β-lactamases and has been shown to enhance the in vitro and in vivo activity of selected penicillins.[101] An organism designated Streptomyces cattleya elaborates thienamycin, whose isolation was greatly complicated by instability.[102] Thienamycin has low MIC values against bacteria, including problem organisms such as P. aeruginosa, S. marcescens, and B. fragilis and is very effective in curing experimental mouse infections.[103] Thienamycin has been shown to contain a 1-carba penem system (15).[104]



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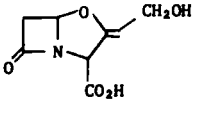

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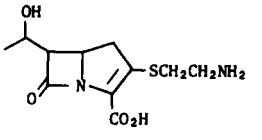

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Chapter 12. Aminocyclitol and Other Antibiotics

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General - Abstracts appeared for the 16th Interscience Conference on Antimicrobial Agents and Chemotherapy,[1] a major conference covering virtually every aspect of antibiotic research. In another meeting for which abstracts were published,[2] symposia were held relating to clinical and experimental therapeutic studies, antibiotic susceptability, and resistance. A fermentation symposium held in Berlin[3] included a session covering various aspects of antibiotic research. The eighteenth volume of *Methods in Enzymology*[4] was devoted to a review on enzymes involved in antibiotic biosynthesis.

In specific antibiotic research areas, development of improved β-lactams (see Chapter 11) and work on aminocyclitol antibiotics represented a significant portion of the effort. Advances were also made in areas of interest on other antibiotics of importance in medicine or agriculture, such as the macrolides, tetracyclines, lincomycins, ansamycins, novobiocins, polyethers, and peptides.

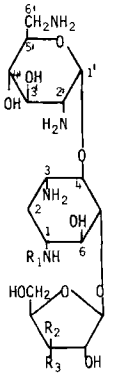
Aminocyclitols - This category of antibiotics includes both aminoglycosides and other antibiotics which contain aminocyclitol moieties but no aminosugar. Control or alteration of resistance development and the moderation of toxic effects are the major areas of research interest. A number of newer aminocyclitols, such as amikacin, sisomicin, verdamicin, netilmicin, were undergoing clinical examination, while tobramycin and amikacin became available for medical use.[5,6]

Inactivating enzymes again received their share of attention. Aminoglycoside 3'-phosphotransferase II was purified[7] and a new 4'-O-nucleotidyltransferase was found to be mediated by a plasmid from a *Staphylococcus aureus* strain.[8] A novel 6'-N-acetyltransferase from a *Pseudomonas aeruginosa* is characterized by reduced activity towards butirosin and amikacin.[9] Another *Pseudomonas aeruginosa* produces a new 3-N-acetyltransferase which has a wider substrate range than those described heretofore.[10] Evidence was recently presented indicating gentamicin acetyltransferase I catalyzes acetylation of amines at the 3-, 2'- and 6'-positions.[11] Clinical resistance of *Pseudomonas aeruginosa* to lower gentamicin levels was ascribed to alteration of the membrane transport function which is not plasmid mediated.[12] Speedy detection of aminoglycoside susceptibility in monomicrobic urinary tract infections involved the use of the urine specimen itself as the inoculum for disc diffusion testing.[13]

The use of D-glucaro-δ-lactam (K salt) reduced the nephrotoxicity of aminoglycosides in rats.[14] Postmortem studies revealed the accumulation of gentamicin and amikacin in human kidneys.[15] Certain aminoglycosides

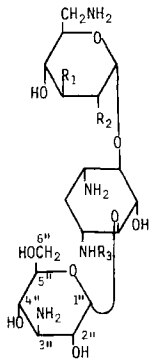
appeared to induce mistranslation in eukaryotic ribosomes[16] while others did not.

In a review on the biosynthesis of aminocyclitols it was pointed out that glucose generally supplied the carbon backbone of each moiety.[17] Mutants of *S. fradiae* and *S. rimosus* incorporated 2,6-dideoxystreptamine into 6-deoxyneomycins and 6-deoxyparomomycins, respectively.[18]

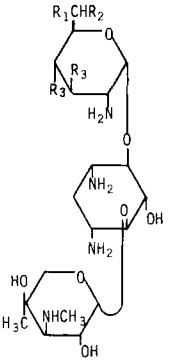


	R_1	R_2	R_3
Ribostamycin	H	H	OH
Butirosin A	*	OH	H
Butirosin B	•	H	OH

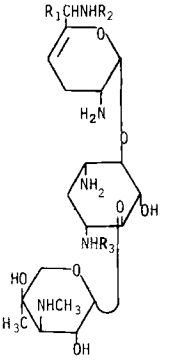
*-$COCHOHCH_2CH_2NH_2$



	R_1	R_2	R_3
Kanamycin A	OH	OH	H
Amikacin	OH	OH	*
Tobramycin	H	NH_2	H



	R_1	R_2	R_3
Gentamicin C_1	CH_3	$NHCH_3$	H
Gentamicin C_{1a}	H	NH_2	H
Gentamicin C_2,C_{2a}	CH_3	NH_2	H
JI-20A	H	NH_2	OH



	R_1	R_2	R_3
Sisomicin	H	H	H
Verdamicin	CH_3	H	H
Netilmicin	H	H	C_2H_5
G-52	H	CH_3	H

Gentamicin remained the standard for the measurement of progress in aminocyclitol chemotherapy. The uses of radioimmune assays[19,20] and an enzymatic assay with gentamicin acetyltransferase I[21] for monitoring serum concentrations were recommended. In contrast to some human studies, cephalothin protected rats against gentamicin nephrotoxicity.[22] Gentamicin-minocycline synergism was described for a number of pathoens.[23] A gentamicin phosphotransferase of staphylococcal origin, also inactivating sisomicin, kanamycin, and tobramycin, was described.[24] The biosynthesis of the gentamicin C-complex from minor gentamicin components by a blocked *Micromonospora purpurea* mutant was studied.[25] Gentamicin B and JI-20A[26] as well as gentamicin X_2[27] were synthesized from a garamine derivative while several analogs incorporating desosamine into the kanamycin and gentamicin series were prepared.[28]

Amikacin was found to have good activity against many isolates resistant to gentamicin, kanamycin, tobramycin, and sisomicin, due to its stability towards inactivating enzymes.[29] Synergism was observed with carbenicillin *vs. Pseudomonas aeruginosa*.[30] Speedy radioimmune assays with ^{125}I-amikacin were more sensitive than bioassays.[31] The efficacy of amikacin was described as comparable to kanamycin or gentamicin in neonatal bacterial diseases.[32]

Sisomicin was equivalent to gentamicin, protecting mice against five infecting organisms.[33] Serum levels in healthy humans were equivalent to those for gentamicin or tobramycin, but excretion was slower[34] while 18 clinical cures were effected in a series of 29 sensitive and resistant Gram-negative urinary tract infections.[35] A new, highly sensitive radioimmune assay for sisomicin is available.[36] In the presence of (Me-^{14}C-)-L-methionine, the sisomicin fermentation produced two 4"-C-desmethylsisomicins and a gentamicin A-like substance.[37] *Micromonospora rhodorangea* transformed sisomicin to gentamicin C_{2b}.[38]

Netilmicin (N-ethyl sisomicin formed by reductive alkylation)[39] was more active than sisomicin against a series of clinical isolates and active against 97% of the sisomicin-resistant organisms.[40] In a comparative study with tobramycin, gentamicin, sisomicin and amikacin, netilmicin was most active against *E. coli* and *K. pneumoniae*.[41] Gentamicin-resistant *Klebsiella* and *Citrobacter* isolates were sensitive to netilmicin, but 73% of the gentamicin-resistant *Pseudomonas* and *Providencia* strains were resistant. In one study, netilmicin was active against gentamicin-resistant *Enterobacteriaceae* that produce adenylating enzymes, but inactive against those producing acetylating enzymes.[42] Compared to gentamicin, netilmicin is less nephrotoxic in rats,[43] less toxic chronically in cats, but more toxic acutely in mice.[44] Another initial clinical study reported it to be effective and safe.[45] Its synergism with penicillins was reported.[46] A radioimmune assay is available.[47]

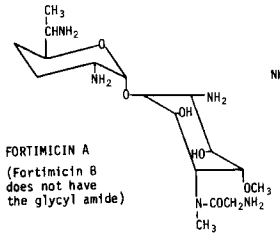
Clinical reports on tobramycin continued to describe cures in a variety of Gram-negative infections.[48] A radioimmune assay was described.[49] Initial explorations into ^{15}N-NMR spectra of several of the nebramycin components suggested a promising tool for following aminoglycoside modification.[50]

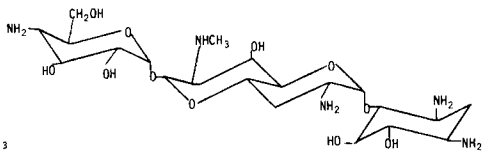
Spectinomycin continued to be effective in the treatment of resistant strains of *N. gonorrheae*[51] including the new β-lactamase producing strains.[52] In cell-free systems, it inhibited peptide chain initiation.[53] It had a very low sensitization rate in guinea pigs.[54] The biosynthesis of spectinomycin from (6-^{13}C)glucose was reported.[55]

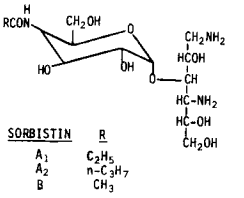
Apramycin showed effect against enteric disease in cattle.[56] Its structure was confirmed[57] by x-ray along with ^{13}CMR assignments.[58]

Streptomycin appeared to enter *Pseudomonas aeruginosa via* active transport.[59] In resistant *Pseudomonas aeruginosa* K102, reduced membrane permeability as well as decreased affinity of streptamine to the ribosome were plasmid-mediated.[60] A 3"-deoxystreptomycin, active *in vitro* against streptomycin-resistant bacteria, was synthesized.[61]

New aminoglycosides with their producing organisms were G-52 (*Micromonospora zionenis*),[62] AB-74 (*Streptomyces aquacanus*),[63] sorbistins A_1, A_2, B (*Pseudomonas sorbicinii*),[64,65] and fortimicins A and B (*Micromonospora olivoasterospora*).[66,67]

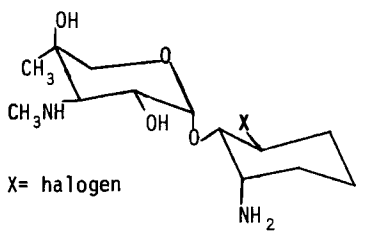




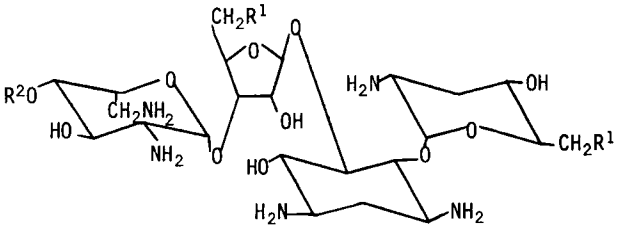


The inclusion of the sorbistins in this category of antibiotics suggests that the name aminocyclitol could be replaced with aminopolyol.

Additional synthetic activities include the total synthesis of ribostamycin,[68] the semisynthesis of SCH 22591[69] with improved activity over gentamicin, and the semisynthesis of 6',5",6""-triamino-6',5",6""-trideoxylividomycin A (I) and 6',5"-diamino-6',5"-dideoxylividomycin B (II).[70] A method for selective N-acylation of gentamicin antibiotics was described.[71]



SCH-22591



I $R^1=NH_2$ R^2=6-Amino-6-deoxy-α-D-mannopyranosyl

II $R^1=NH_2$ $R^2=H$

Macrolides - Clinical trials are underway for erythromycin A, 11,12-cyclic carbonate,[72] which is said to be more active than erythromycin A. Josamycin was tolerated better than erythromycin in fasting patients, while plasma concentrations, half lives, and elimination constants were equivalent.[73] Josamycin was less active than rosamicin or erythromycin A against aerobic cocci while clindamycin was more active than all three against anaerobes.[74,75]

Two new minor macrolides were discovered, 10,11-dihydropicromycin[76] and 3"-de-O-methyl-2",3"-anhydrolankamycin.[77] In addition, *Micromonospora chalcea* produced a family of 8 juvenimicins along with the everninomicins.[78] Of these, five have been isolated. One was rosamicin, but the other four

are new macrolides with 16-membered lactones and the sugar, desosamine.[79] *Streptomyces antibioticus* converted erythronolide A oxime to 3-O-oleandryl-5-O-desosaminylerythronolide A[80] which was more active than oleandomycin but weaker than erythromycin. The aglycone of leucomycin A_3 was isolated, the first such instance in the 16-membered series.[81] Relative to the total synthesis of macrolides, *bis*-2-imidazole disulfides were used for cyclization of hydroxy acids to macrolactones.[82]

Tetracyclines - An extensive review of the chemistry and biochemistry of the tetracyclines, including a structure/activity discussion appeared.[83] The newly synthesized 9-methyltetracycline was more active than tetracycline which in turn was more active than novel 7-methyltetracycline.[84] In a double blind clinical trial, doxycycline once daily was equivalent to 4 daily doses of tetracycline.[85] It was reported that a plasmid-mediated transfer of resistance from 018 *E. coli* to 088 *E. coli* occurred 202 days after administration of tetracycline in a human.[86]

Clindamycin-Lincomycin - New *in vitro* studies appeared in which the sensitivity of anaerobes to clindamycin compared favorably to chloramphenicol and penicillin[87] as well as josamycin and everninomicin B.[88] Antagonism was reported between clindamycin and aminoglycoside antibiotics.[89] Lincomycin had two effects on polytoxic staphylococci,[90] *viz.*, the well-known inhibition of protein synthesis (toxins and enzymes accumulated neither in the cells nor the medium) and also an effect on protein release through the cytoplasmic membrane (certain enzymes and toxins accumulated in the cells). A model study system using polymixin to release the proteins was described. Summaries appeared describing the clinical usefulness of clindamycin in 627 pediatric cases involving β-hemolytic streptococci,[91] in 96 patients with anaerobic wound infections,[92] and in 60 cases of acne.[93] In hamster[94] and mouse[95] models for clindamycin colitis, fecal flora were suggested to have roles. Radioimmune[96] and thin layer chromatographic[97] assays were published for clindamycin.

Ansamycins - The structures, reactions, physical properties and biosynthesis of these antibiotics were reviewed.[98] Protostreptovaricins I-IV were described as precursors of the streptovaricins.[99] Also, the biosynthetic sequence damavaricin D → SvD → SvC → SvB, SvJ was determined.[100] The isolation and structure of actamycin was reported.[101] An immunosuppresent effect in mice of rifamycin and streptovaricin analogues correlated with toxicity but not with *in vitro* activity.[102] The plasma decline of ethynylestradiol after administration of rifamycin was quantified in humans.[103]

Novobiocin - It was reported that the previously reported anomeric α-configuration for noviose in novobiocin is incorrect.[104] Methyl β-novioside was synthesized by a stereoselective synthesis from a non-sugar precursor and the configuration was opposite to that previously reported in the literature. However, the corresponding sugar coumerose, in the related antibiotic coumermycin A_1 was found to be in the α-configuration according to x-ray crystallographic studies.[105] The C-13 NMR spectral assignments have been made.[106] Semiconservative DNA replication in

toluenized *E. coli* cells was strongly inhibited by novobiocin while ATP-independent DNA repair and protein synthesis were not affected.[107] Similar activity for coumermycin A_1 was characterized.[108]

Polyethers - Again there was considerable activity in the field of these antibiotics which interfere with the normal function of microbial membranes. Monensin is now being used as a cattle feed additive.[109] Four new polyethers were reported: lonomycin[110,111] CP-38,295,[112] narasin[113,114] and K-41.[115] The structure was determined for laidlomycin[116] and a procedure for the isolation of alborixin was outlined.[117]

Peptides - A number of new peptide antibiotics were isolated and described in 1976. SCH-18640, produced by *Micromonospora arborensis*, was characterized as a member of the thiostrepton group.[118] *Bacillus brevis* elaborated brevistin, active *in vivo vs.* Gram-positive organisms. It is a cyclic depsipeptide containing a fatty acid.[119,120] A group of new broad spectrum cyclic peptides containing C9 to C11 fatty acids was named the octapeptins.[121] The broad spectrum cyclic peptide B-43,[122] produced by *Bacillus circulans*, was related to polypeptin, the structure of which was elucidated recently.[123] The isolation and structure of the antifungal and immunosuppressive cyclosporin A was reported.[124] *Bacillus pumilus* produced the basic peptide 339-29 which was active against Gram positive organisms.[125] *Actinoplanes garbadinensis* elaborated the linear peptide gardimycin which inhibited cell-wall synthesis.[126,127] The cerexins, including two new ones, cerexin C and D, were shown to comprise combinations of 10-unit linear peptides partially esterified to C10 and C11 hydroxy acids.[128,129] The isolation and structure of feldamycin, a dipeptide was reported.[130,131] Sporamycin was isolated and characterized.[132]

Structures and revisions were reported: alamethicin (now a linear peptide),[133] suzukacillin,[134] actinoxanthin,[135] I-2743-C (an antimycin),[136] bottromycin A_2,[137] mycosubtilin,[138] iturin (an active component of bacillomycin B),[139] and echinocandin B.[140] Structures of thiostrepton and siomycin A were finalized using ^{13}C-NMR,[141] while the griseoviridin,[142] capreomycin[143] and quinomycin[144] structures were revised.

The site of activity for polymixin B was located at the bacterial membrane[145] and the dipeptide enomycin inhibited the initial phase of protein synthesis.[146]

Chloramphenicol - L-*Threo*-chloramphenicol, an isomer which does not inhibit mitochondrial protein synthesis, did exert an effect on bone marrow in rats.[147] Previous studies had related the two effects. A resistance factor elaborated by *Pseudomonas aeruginosa* K-102 was thought to control the function of membrane permeability of the cells.[148]

Miscellaneous Antibiotics - Miscellaneous new antibiotics from various sources were described in the literature in addition to those discussed above. These are summarized very briefly in Table 1.

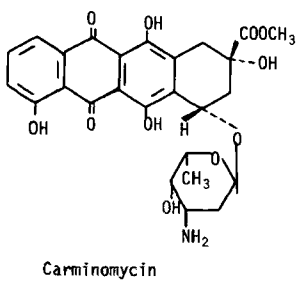
New structures were published or revised for other miscellaneous antibiotics such as carminomycin,[178] enterocin,[179] ilicicolin H,[180]

Table 1: Additional New Antibiotics Reported in 1976.

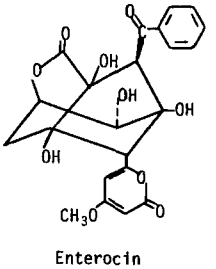
Antibiotic	Producing Organism	Activity*	Reference
Chlorocarcin A,B,C	*Streptomyces lavendulae*	G+, AT	149
Mimosamycin	*Streptomyces lavendulae*	G+, AF	149
1294 B-2	*Nocardia formica*	AB, AF	150
Laterosporamine	*Bacillus laterosporus*	G+, G-	151
KM-214	*Bacillus aurantinus*	G+	152
Maduramycin	*Actinomadura sp.*	G+	153
Efrotomycin†	*Streptomyces lactamdurans*	G+	154, 155
Neothramycin A,B†	*Streptomyces sp.*	AB,AF,AT	156
K-52A	*Streptoverticillium roseoverticillatum*	G+, G-	157
Nanaomycin D†	*Streptomyces rosa*	AB, AF	158
Griseusins A,B†	*Streptomycin griseus*	AB, AF	159
Ficellomycin	*Streptomyces ficellus*	G+	160
2-Methyl-L-arginine	*Streptomyces sp.*	G+, G-	161
Malformin C†	*Aspergillus niger*	-	162
Compactin†	*Penicillium brevicompactum*	AF -	163
1-Deoxy-D-*threo* pentulose†	*Streptomyces hygroscopicus*	*M. avium*	164, 165
P-3355	*Streptomyces amylovorus*	G+, G-	166
XK-90†	*Streptomyces sp.* MK-90	G+, G-	167
Indoleacryloiso-nitrile†	*Pseudomonas sp.*	G+	168
Aplasmomycin	*Streptomyces griseus (Marine)*	G+, *mycob.*	169
Asukamycin	*Streptomyces nodosus*	G+	170
U-43120	*Streptomyces paulus*	AB, AT	171, 172
Nikkomycin†	*Streptomyces tendae*	AF	173
5,6-Dihydro-5-azathymidine†	*Streptomyces platensis*	G-, AV	174
Tirandamycin B†	*Streptomyces flaveolus*	G+, AF	175
Rancinamycins†	*Streptomyces lincolnensis*	G+, G-	176
Lysolipin I,X†	*Streptomyces violaceoniger*	G+	177

*G+, G- (Gram positive, negative); AB, antibacterial; AF, antifungal; AT, antitumor; AV, antiviral.
†Structures are published for the indicated antibiotics.

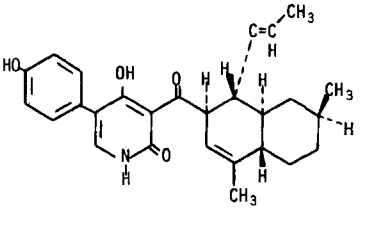
kalafungin,[181] conocandin[182] and methylenomycin B.[183] The red alga *Chondria californica* produces at least six polysulfides which are responsible for the antibiotic activity of this culture.[184] The antifungal antibiotic hamycin was found to be a mixture of 4 components, A, B, C, and D and hamycin A was assigned a structure.[185] NMR studies (^{13}C)



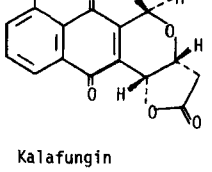
Carminomycin



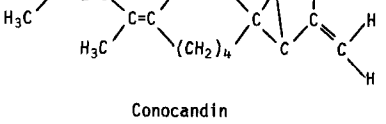
Enterocin



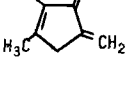
Ilicicolin H



Kalafungin



Conocandin



Methylenomycin B

showed that while erythromycin exists in the keto form in solution, amphotericin B, nystatin, tetrin A and B, pimaricin, and lucensomycin all exist in a hemiketal form.[186] Total syntheses were reported for nectriapyrone,[187] pyrenophorin,[188] nonactic acid[189] and marasmic acid.[190] Syntheses were also reported for showdomycin,[191] melrosporus,[192] and 5,6-dihydro-5-azathymidine.[193,194]

In addition to a review of the biological activity of nucleoside antibiotics,[195] studies on the mechanism of action of miscellaneous antibiotics appeared in the literature. Leucinostatin, a peptide, acted on membrane phospholipid.[196] Inhibition of fatty acid synthesis and polyketide synthesis accounted for the activity of cerulenin,[197] while tirandamycin interfered with RNA polymerase, thus inhibiting transcription.[198] Evernomicin B altered the cytoplasmic membrane in a non-bactericidal manner leading to inhibition of metabolite uptake and DNA replication.[199] Bicyclomycin affected lipoprotein synthesis.[200] A group of polyenes which are not effective against normal rod forms of *Escherichia coli* were active against L-forms of *E. coli* W1655F, supporting the existence of a cell wall barrier to these substances in prokaryotic cells.[201] Resistance to virginiamycin M by a *S. aureus* strain was due to acetylation of the secondary hydroxyl function.[202]

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Chapter 13. Antineoplastic Agents

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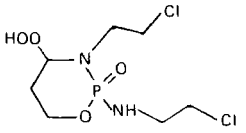
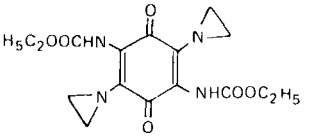
Introduction - The history, development, mechanism of action, pharmacology and clinical status of several important cancer chemotherapeutic agents were reviewed during 1976. Symposia treating these topics were devoted to cyclophosphamide,[1] the nitrosoureas,[2] DTIC[3] and adriamycin.[4] The status of platinum complexes as antitumor agents was also described.[5] The history of the National Cancer Institute (N.C.I.) plant products program was detailed along with examples of the types of active materials which have been isolated.[6] The organization and function of the N.C.I. Registry of Experimental Cancers which has more than 21,000 accessions was reported[7] as was a review of the use of anticoagulants in the treatment of cancer.[8] The state-of-the-art in the study of anticancer drug-ionizing radiation interaction and potentiation was also reviewed.[9]

Alkylating Agents - Levorotatory cyclophosphamide was recovered from the urine of patients given the racemic drug.[10] This indicated a preferential metabolism for the dextro form. When the two isomers were prepared and tested, the therapeutic index of the levo form was found to be twice that of the dextro. Direct ozonation of cyclophosphamide, ifosfamide and trofosfamide gave the 4-hydroperoxy derivatives.[11] The ifosfamide analogue (1) was the most active of the three giving 100% 30-day survivors in L1210 leukemia tests. When the enzymes involved in activating conjugates of p-hydroxyaniline mustard were studied, the results were consistent with a relationship between HeLa cytotoxic activity and cell membrane alkaline phosphatase activity for the O-phosphate derivative.[12] The redox potentials of a number of benzo- and naphthoquinone bioreductive alkylating agents were correlated with Sarcoma 180 (S-180) activity.[13] The most easily reduced compounds were the least active. Aziridinyl benzoquinones were prepared as CNS antitumor agents. Among a number of very active compounds, AZQ (2) gave cures in the murine ependymoblastoma brain tumor system.[14]



1



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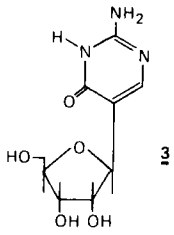
Carbazilquinone was very effective against the intramuscular (i.m.) Ehrlich ascites tumor when used with local hyperthermia (37-41°) and temporary interruption of blood flow.[15] Mature ribosome formation was blocked by BCNU indicating that the effectiveness of this drug as a cell growth inhibitor may be due to the inhibition of protein synthesis.[16] A new nitrosourea glucoside had activity which was comparable to that of

CCNU in the L1210 system.[17] An adamantyl TEPA analogue possessed both intraperitoneal (i.p.) and intracerebral (i.c.) L1210 activity.[18]

Folic Acid Antagonists - Methotrexate (MTX), folinic acid and ara-C were used sequentially with positive results in patients who had become resistant to conventional chemotherapy.[19] The AT-3000 MTX resistant line of S-180 cells were found to have at least 150 times more dihydrofolate reductase than MTX sensitive cells.[20] Similar data were obtained with MTX resistant hamster cells.[21]

Nucleosides - The stability of 5-azacytidine (5-AC) in a number of solutions was determined. The half-life in human plasma at 37° was 5 hours.[22] A reduced analogue of 5-AC, 5,6-dihydro-5-azacytidine, was completely stable in aqueous solution and possessed L1210 activity which was comparable with 5-AC.[23] Pseudoisocytidine (3), a C-nucleoside which might be considered as an analogue of 5-azacytidine (5-AC), is as active as 5-AC in vivo, hydrolytically stable, and not deaminated by cytidine deaminase.[24] Streptomyces platensis var. clarensis was found to produce pseudouridine, 1-methylpseudouridine and 5-methyl-5-aza-2'-deoxyuridine.[25] The in vivo P388 antitumor activity of cordycepin (3'-deoxyadenosine) was potentiated by 2'-deoxycoformycin (4), a deaminase inhibitor.[26] The same compound potentiated the L1210 activity of adenine arabinoside, which is inactive as a single agent, by inhibiting adenosine deaminase.[27]



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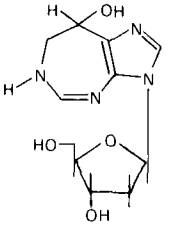

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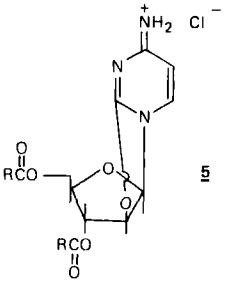


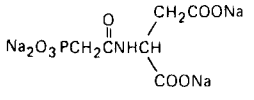
5

Among several water-soluble derivatives of ara-C prepared for L1210 testing, the activity of the 5-adamantoyl hydrochloride salt was almost as good in oral tests as the activity of the parent, ara-C, in i.p. tests.[28] The first example of an irreversible thymidylate synthetase inhibitor was discovered when 5-iodoacetamidomethyl-2'-deoxyuridine-5'-phosphate was tested.[29] Evidence was presented for a covalent sulfide linkage between 5-FUdR-5'-phosphate, 5,10-methylenetetrahydrofolate and thymidylate synthetase in a ternary complex.[30] D-Galactosamine was used to induce depletion of UTP and synergistically enhance the growth inhibition of liver tumors by 3-deazauridine.[31] Several trifluoromethyl analogues of thymidine were compared in vitro. 5-Trifluoromethyluridine and its 5'-phosphate were the most active materials.[32] In a series of papers,[33] acylated derivatives of anhydro ara-C (5) were described which produced long term L1210 survivors. Replacement of the heterocyclic 3-NH function with oxygen in uridine produced a nucleoside with modest in vitro activity.[34] The cytotoxicity of inosine dialdehyde to L1210 cells

was greatly reduced by amino acids and plasma proteins because of complexation or cross-linking reactions.[35] Although 8-aza-6-thioguanine was active in vitro, a group of 6-substituted-8-azaguanine nucleosides were generally inactive compounds.[36] Direct fluorination of uracil, cytosine and their nucleosides was achieved using trifluoromethyl hypofluorite.[37]

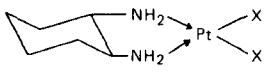
Amino Acids, Proteins and Enzymes - The inactivation which occurs when neocarzinostatin is exposed to serum was found to be related to the degradation of the drug to smaller molecules. Since sulfhydryl reactive reagents, eg. N-ethylmaleimide, protected neocarzinostatin from this decomposition reaction, it was suggested that the degradation was caused by serine type proteinases with mercapto group involvement.[38] Bestatin, (2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine, binds with cell surface aminopeptidases. This compound augmented the immune response and suppressed tumor growth caused by a second inoculation of Ehrlich carcinoma cells.[39] Crystalline glutaminase-asparaginase enzyme was prepared from Pseudomonas 7A. Unlike the enzyme obtained from Acinetobacter, the Pseudomonas product was active against both ascites and solid rodent tumors. It also had a plasma half-life which was longer than that of the Acinetobacter material.[40]

Synthetic Agents - PALA (6), a transition-state inhibitor of aspartate transcarbamylase, was found to be very active against the subcutaneous (s.c.) Lewis lung and i.p. B16 melanoma tumor models.[41] Antitumor active acridines were used to study the effect of tumor implantation site on the choice of the most active congener in a series.[42] A different optimum compound was found with s.c., i.p. and i.c. L1210 implantation. For good activity against a tumor implanted in a region remote from the i.p. drug administration site, an optimal agent must be more lipophilic than one which is optimum for an i.p. implanted tumor. The discovery that the enantiomers of ICRF 159 were five times more soluble than the racemic mixture helped overcome a formulation problem.[43] 2-Formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone was synthesized to react specifically with pyridoxyl phosphokinase and was active in the L1210 and S-180 systems.[44] Arylsulfonylhydrazones of 2-formylpyridine N-oxide exhibited activity against several tumor systems. The N-oxide function was generally an activity requirement and substitution of carbonyl for sulfonyl caused a loss of activity in these inhibitors of DNA and RNA synthesis.[45] While DTIC is light sensitive and prone to decompose to a toxic diazonium ion, the aryltrialkyltriazenes are antitumor-active and non-photosensitive. Evidence was obtained that only the compounds which can be metabolized to N^3-monomethyltriazenes are active.[46] Boron analogues of betaine (7a) and its N-ethylamide (7b) were active in the Ehrlich and P388 tumor models.[47] The hydrochloride but not the methiodide



6

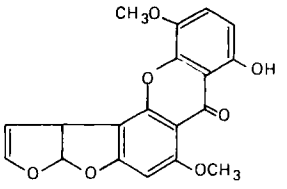
7a, R = OH
7b, R = NHCH3



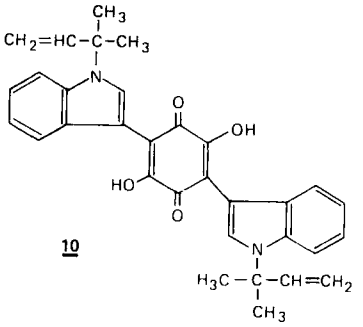
8a, X = Cl
8b, X = NO3

salt of 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide was active *in vivo* against the murine C1300 neuroblastoma.[48] Carbamoyl mycophenolic acid[49] was orally active against the L1210 tumor giving an increase in lifespan (ILS) of 64%. The antitumor activity of positional and structural isomers of coralyne was explained in terms of N-O-O triangulation.[50] DL-α-Hydrazino-δ-aminovaleric acid is S-180 active and a potent ornithine decarboxylase inhibitor.[51] Janssen R17934, a benzimidazole carbamate, interfered with the structure and function of microtubules (see Chapter 15). Malignant cells were more susceptible to this effect than normal cells, *in vivo*.[52] Tamoxifen (ICI 46,474) was active against DMBA induced mammary tumors[53] while β-retinoic acid analogues reduced the extent and incidence of methylcholanthrene induced tumors.[54] A large number of *cis*-amineplatinum compounds were screened for activity in the L1210 system.[55] Dichloro-(1,2-diaminocyclohexane)platinum (II) (8a) was the most active compound (ILS 139%). A later study showed that dinitrato(1,2-diaminocyclohexane)-platinum (II) (8b) gave ILS values as high as 281% with 30% survivors in the same tumor model.[56] *Cis*-diamminodichloroplatinum (II) was found to cause multiple cellular and subcellular effects in chick embryo fibroblasts.[57] Rhodium (II) acetate gave an ILS of 158% in the Ehrlich ascites system,[58] while i.p. zinc acetate prevented i.p. L1210 tumor growth in a majority of mice treated.[59] Two compounds from a large series of pyrazolo-[3,4-*b*]quinolines were found to be as potent as tilorone as interferon inducers.[60] Calusterone, a clinically-active agent for the treatment of breast cancer, regressed DMBA induced rat adenocarcinomas.[61]

Fermentation Products - Anthracycline research once again dominated the activity in antitumor fermentation products. A practical seven step synthesis for the racemic aglycones of daunorubicin and carminomycin was devised.[62] Several studies attacked the cardiac toxicity problem which is the clinically limiting factor for adriamycin (ADN). Alpha-tocopherol greatly diminished the cardiac toxicity of ADN in mice.[63] ADN cytotoxicity was significantly greater in methotrexate (MTX) resistant L5178Y cells than in the MTX sensitive line.[64] This suggested a potential use



9

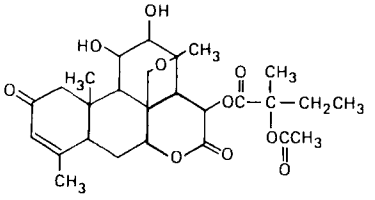


10

for ADN in MTX resistant tumors and the use of these drugs in combination against mixed tumor cell populations. ADN inhibits coenzyme Q_{10} (CoQ_{10}) enzymes which are important in cardiac tissue. The inhibition is prevented by CoQ_{10} administration and CoQ_{10} treatment is suggested as a way

to reduce ADN cardiac toxicity.[65] A difference was noted in the fluorescent properties of cells treated with ADN or AD-32 (N-trifluoroacetyl-adriamycin-14-valerate). ADN fluorescence was most intense in the cell nuclei while AD-32 fluorescence was found mainly in the cytoplasm.[66] The relationship between sugar stereochemistry and cell culture activity was determined for the 1'- and 4'- isomers of ADN and daunorubicin. While an inverted configuration at the 1'-position caused a large decrease in activity, inversion at the 4'-position did not produce much change.[67] Conversion of a lactone to a lactam function in actinomycin D abolished or reduced antitumor activity in three different murine tumor systems.[68] Complexes between actinomycin D and DNA were studied by analysis of circular dichroism spectra.[69] A reversible mitomycin C redox reaction was described which might have a relationship to the mechanism of alkylation of macromolecules by this drug.[70] Macromomycin B increased the immunogenicity of TA3-Ha cells by binding to the cell surface.[71] This compound also was active against the normally refractory Lewis lung tumor model. Several derivatives of 5-methoxysterigmatocystin (9) were prepared and the vinyl ether double bond was found to be necessary for in vivo P388 activity. These compounds, however, are probably potent carcinogens.[72] Asterriquinone (10), isolated from Aspergillus terreus was active against the L1210 and Yoshida tumors. It also provided multiple cures in the Ehrlich ascites system.[73]

Plant Products - Quassimarin (11), a compound isolated from the sap of a Costa Rican plant, gave an ILS value of 75% against P388 leukemia.[74] Lochnericine and horhammericine, two alkaloids isolated from Catharanthus trichophyllus, had ED_{50} values of 1.1 μg/ml in the KB in vitro system.[75] Baccharin (P388 ILS 100%) was shown by x-ray studies to be a macrolide ester fused to a tricyclic ring system.[76] Several of the cannabinoids found in marihuana inhibited in vitro DNA synthesis in L1210, Lewis lung and bone marrow cells. In vivo Lewis lung activity was also observed.[77] Simultaneous injection of citrate prevented the severe hemolysis observed when dogs and monkeys were administered intravenous solutions of ellipticine.[78] Seven colchicine analogues were tested for their ability to inhibit



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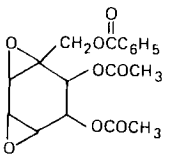

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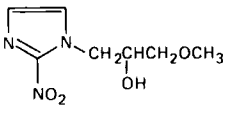
mitosis in HeLa cells and inhibit tubulin polymerization in mouse brain extracts. Structure-activity relationships suggest that while phenyl and methoxy groups were necessary, the seven member tropone ring was not required.[79] While the rate of binding of colchicine to tubulin was enhanced by certain anions, no similar effect was noted with vinblastine or podophyllotoxin leading to the conclusion that anions have a local

effect at or near a colchicine binding site.[80] A synthesis of (±) crotepoxide (12), a Walker and Lewis lung active, was devised.[81]

Radiosensitizing Agents - Agents which sensitize tumor cells to the lethal effects of ionizing radiation are being actively pursued. Two known radiosensitizers, metronidazole and nitrofurazone, were shown to be cytotoxic to Chinese hamster cells in the absence of radiation, but only under hypoxic conditions.[82] The x-ray requirement for antitumor activity in a murine anaplastic carcinoma was reduced when metronidazole was administered orally before irradiation.[83] RO-07-0582 (13), a compound with radiosensitizer properties, has a four-fold higher association with hypoxic cells in culture than with cells incubated in air.[84] 5-Thio-D-glucose killed hypoxic murine mastocytoma cells while only suppressing the growth of those exposed to air.[85] It was suggested that this compound may be a useful adjuvant to radiotherapy.

Immunotherapeutic Agents - C. parvum protected mice best against a lymphosarcoma when the cells and agent were injected i.v. In contrast, i.p. treatment was best with a mammary carcinoma.[86] DNA significantly potentiated the antitumor effects of adriamycin and actinomycin D. The DNA can be complexed with the drug or given up to four hours before or after drug treatment. These results are consistent with DNA stimulation of the immune response system.[87] Daily administration of mouse interferon preparations to AKR mice with a lymphoma increased average survival times by 100%. This result compares favorably with the results obtained with standard anticancer drugs.[88] DTIC caused an immunologic alteration of L5178Y lymphoma in vivo resulting in long term survivors after treatment with BCNU.[89] Pyran co-polymer was as effective as BCG in inhibiting Lewis lung carcinoma and MCA 2182 sarcoma.[90] Phytohaemagglutinin could be used to stimulate, as well as inhibit, tumor growth in mice with Harding-Passey melanoma.[91] A fused thiazole acetic acid (WY 13,876) (14), a levamisole analogue, showed low level Lewis lung activity.[92,93] This compound was non-cytotoxic in cell culture, but was synergistic with 5-FU and cyclophosphamide in vivo.[93]



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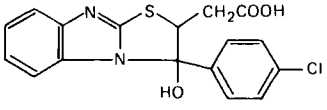

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Miscellaneous Agents and Techniques - When actinomycin D (ADX) was incorporated into lipid vesicles, the drug was carried into cells which were previously resistant to the drug because of poor penetration.[94] A five-fold increase in drug concentration was achieved in the resistant DC-3F/ADX cells relative to treatment with ADX alone. When ara-C was entrapped in phospholipid vesicles, activity was significantly greater than for the drug alone.[95] Supranormal temperatures (42.5-43°) for eight hours had a greater lethal effect on human tumor cells than on normal cells.[96] The effect of local hyperthermia as cancer therapy on Yoshida

sarcoma in rats showed that 42° cured the tumor but 40° enhanced dissemination of the tumor and decreased survival times.[97] By the use of trapping experiments, singlet oxygen was shown to be the *in vitro* active agent produced by red light in the presence of hematoporphyrin.[98] Quantitative structure-activity relationships were developed for the inhibition of dihydrofolate reductase by triazine antifols.[99] The antitumor activity and toxicity of mono-substituted phenyltriazenes correlated quantitatively with sigma values. A similar relationship for the hydrolysis rates suggested that triazene hydrolysis to a diazonium cation in the cell may be involved in the cytotoxic mechanism.[100]

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Chapter 14. Biosynthesis of Antibiotics

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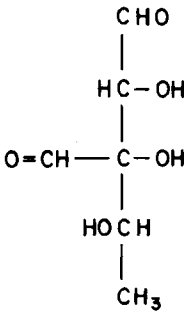
A resurgence of interest and accomplishment in the field of biosynthesis of natural products - including antibiotics - has occurred. The powerful methods for determination of a new structure with small amounts of material (mass and nmr spectroscopy, etc.) together with the development of ^{13}C nmr spectroscopy for following the pattern of incorporation of precursors into these structures is one major reason. The subject of biogenesis of antibiotics has not been dealt with specifically in this series and this brief review will include some literature from earlier years than 1976.

Several annual reports appear [1,2]. There are three recent volumes with numerous articles on biosynthesis [3,4,5]. The regulation of antibiotic biosynthesis has been reviewed [6].

The streptamine or 2-deoxystreptamine moieties of the *AMINOGLYCOSIDE* antibiotics are formed from D-glucose, possibly via myo-inositol, and interestingly two different pathways seem to exist. Streptidine and actinamine (spectinomycin) are formed by a pathway in which C-6 of the glucose becomes C-6 of the cyclitol, whereas with deoxystreptamine (neomycin etc.) C-6 becomes C-2 [7,8,9,10]. The biosynthesis of the L-streptose (1) moiety of streptomycin (2) depends on dTDP-D-glucose which by a sequence of reaction involving a rearrangement (C-3 of glucose to C-3 formyl of streptose) gives dTDP-dihydro-L-streptose. This sugar derivitive is transferred to streptidine-6-phosphate to give the phosphorylated disaccharide and that in turn accepts activated N-methyl-L-glucosamine to yield dihydrostrepto-



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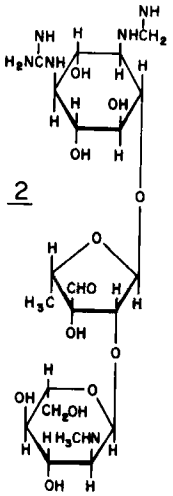

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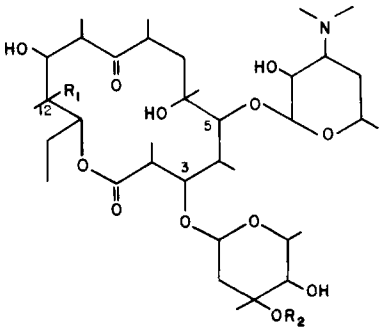
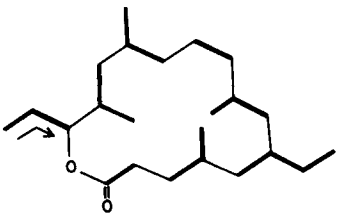


3 $R_1 = R_2 = H$
4 $R_1 = H, R_2 = Me$
5 $R_1 = OH, R_2 = H$
6 $R_1 = OH, R_2 = Me$

mycin phosphate. The hydroxymethyl branch group of the dihydrostreptose moiety is oxidized either before or after addition of the glucosamine fragment when streptomycin phosphate is the product. A specific 6-phosphatase completes the synthesis of either antibiotic [11,12,13,14,15,16].

Considering *MACROLIDE* antibiotics, erythromycin D (3), an obligate precursor of the B, C and A forms was described [17]. Transmethylation [18] from S-adenosyl-L-methionine (SAM) gives the B form (4) or alternately hydroxylation by a membranous, non-cytochrome P450-dependent enzyme [19] gives the C (3) form. Erythromycin C on transmethylation, apparently by the same enzyme, gives erythromycin A (6). Biogenesis of the lactone of erythromycins B and D depends on a soluble cytochrome P450-dependent mixed function oxydase [20]. Further oxidative metabolism of erythromycin A gives the E form with an orthoester link between the oxidized neutral sugar and the lactone [21]. Using ^{13}C nmr the biosynthesis of picromycin has been studied and the results confirm that a malonate building block is used in the synthesis [22]. Several novel 14-member macrolides have been reported either from mutant studies [23] or fermentations [24, 25,26]. The biogenesis of both the tylosin (7) (rosamicin, cirramycin etc.) [27,28] and magnamycin (8) (leucomycins, niddamycin, platenomycins, etc) [29,30] classes of 16-member macrolides has been studied. The former are derived from two acetates, five propionates and a single butyrate unit, while the latter arise from five acetates, one propionate, a butyrate and another as yet unidentified 2-carbon fragment.



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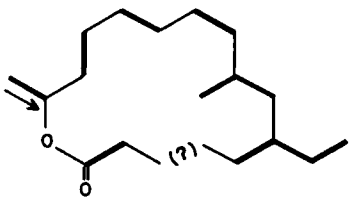

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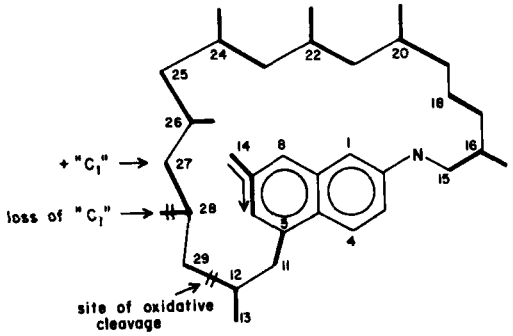
Intensive study of the *ANSAMYCINS* (rifamycins, streptovaricins, tolypomycin, geldanamycin etc.) has indicated a common biogenesis from a polyketide chain initiated by a seven-carbon amino unit of uncertain metabolic origin [31]. Eight propionate/methylmalonate units, two acetate/malonate units (a third for the O-acetyl group at position 25 in rifamycin S) and a methionine-derived methyl group lead to the ansa bridge chain. In rifamycin S an oxygen atom is introduced between positions 12 and 29. In rifamycin W, (9) the progenitor of the S and other members of the complex, the oxygen atom is missing and the W form provides a biogenetic link with the other ansamycins [32, 31, 33, 34, 35].

The biogenesis of monesin, a *POLYETHER* ionophore antibiotic, proceeds from five acetate, seven propionate, and one butyrate molecules. A single methyl group from L-methionine is also present [36]. Narasin, (10) another

polyether ionophore is made from five acetate, seven propionate and three butyrate units, and in the study of its biogenesis [37] evidence for the conversion of propionate to butyrate was presented. The lasalocids are a group of polyether antibiotics which are reported to be made by a polyketide route involving extensive use of both propionate and butyrate building blocks. A unique chromophore is present which resembles 6-methylsalicylate, and this part of the structure seems to be formed from the last part of the precursor polyoxo chain. The homologues of lasalocid described reflect exchange of butyrate/2-ethylmalonate units for propionate/2-methylmalonate building blocks [38,39]. The biosynthesis of the macrotetrolides has also been studied and reports on the utilization of acetate and propionate for the formation of nonactin [40] (11) and homononactinic acid [41] have appeared. The utilization of the optically active nonactinic acids as precursors of the macrotetrolides has also been reported [42].



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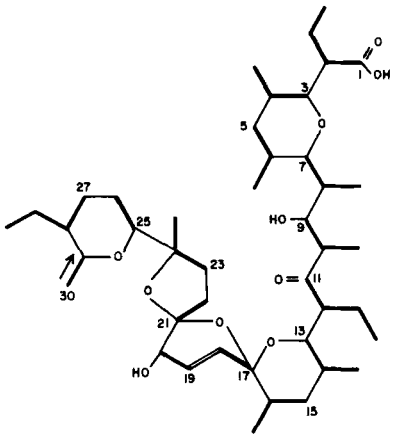

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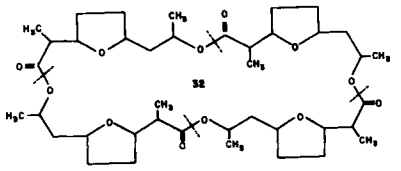
The biogenesis of the *POLYENE MACROLIDE* candicidin (a heptaene) is mostly from acetate and propionate with the additional presence in the structure of p-aminoacetophenone and mycosamine (3-amino-3,6-dideoxy D-mannopyranose) [43]. The biosynthesis of the candidin and candihexin complexes (heptaene and hexaene, respectively) seems to be via sugar-free aglycones which are within the producing mycelium. Glycosylation may take place during secretion of the final products.

Glycosylation seems to occur during the biosynthesis of *TETRACYCLINES* and related antibiotics [44]. Two enzymes, an N-methyl transferase [45] and a tetracycline 5a(11a) dehydrogenase [46] have been described.

The *GLUTARIMIDE* antibiotic streptimidone is formed from seven malonate units and is in itself unusual by having the polyketide synthesis initiated by a malonate unit which is retained intact in the final structure. The derivitive, 9-methylstreptimidone (12) is reported to be made similarly, with a double decarboxylation of the terminal malonate unit. This is

followed by a substitution at this last carbon position with a methyl group derived from methionine, as are the other two methyl groups in both antibiotics [47].



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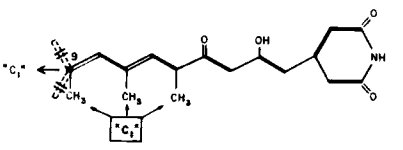

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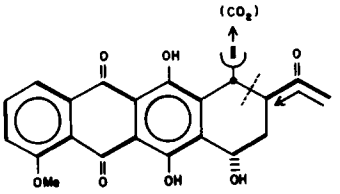

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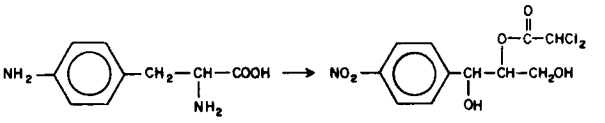
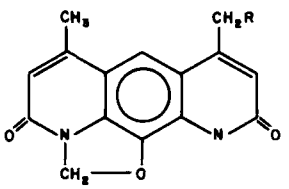


14 15

Coupling of the isoprenoid and polyketide pathways occurs in the biogenesis of siccanin [48] from trans-γ-monocyclofarnesol and orsellinic acid.

Daunomycinone (13) the aglycon of *DAUNOMYCIN* is seemingly made from a single polyketide chain which is unusual both in that a propionate primer is utilized and in the loss of the carboxyl group of the last malonate unit [49].

Several studies of the biosynthesis of *CHLORAMPHENICOL* have led to the conclusion that it is formed via the shikimic acid pathway, specifically from chorismic acid. An arylamine synthetase promotes formation of p-amino-L-phenyl alanine (14) [50,51]. This product is converted to chloramphenicol (15) by oxidation of the amine function to a nitro group, by hydroxylation of the benzylic methylene group, reduction of the carboxyl



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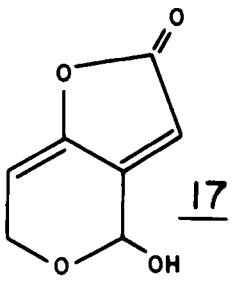

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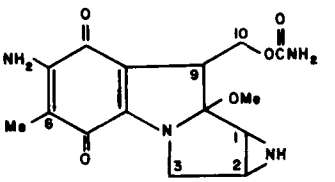


18

group and acylation with dichloroacetate. The sequence of these last steps is not established.

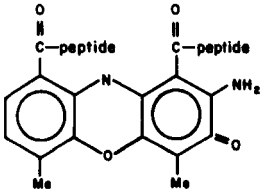
The biosynthesis of *NOVOBIOCIN* has been reviewed [52], and the formation in cell-free extracts of novobiocic acid has been described [53]. The latter can be formed from 3-amino-4, 7-dihydroxy-8-methyl coumarin (B ring) and 4-hydroxy-3(3-methyl-2-butenyl)benzoic acid (A ring) by an enzyme that forms an amide bond between these precursors. Energy (ATP) is required but the mode of activation of the carboxyl group is unknown. Novobiocin itself possesses a sugar (noviose) but its biosynthesis and the formation of the glycosidic link are little studied [52].

The novel new antibiotic *NYBOMYCIN* (16) which possesses both fused pyridoquinolone and angularly fused oxazoline ring systems, is formed from both acetate (external carbons) and shikimate (internal ring) plus a methyl group from methionine [54].

A step in the biosynthesis of *PATULIN* (17) has been clarified by the finding that meta hydroxy benzyl alcohol is hydroxylated to yield gentisyl alcohol which in turn is converted to patulin via the aldehyde [55].

Intensive study of *MITOMYCIN* (18) biosynthesis [56] has not resolved all the problems of its origin. D-Glucosamine provides carbon and nitrogen for the aziridine ring and possibly a C_6N unit joins with a C_7N unit of less certain origin in the biosynthesis. The latter may be related to some intermediate of the shikimate pathway [57] and intermediates of the arginine biosynthetic pathway contribute the carbamoyl group [59,57,60,58].

Many antibiotics contain *OLIGOPEPTIDE* sequences or are made up entirely of amino acids in peptide linkage. Many of the acids are of the D-configuration and some are of unusual structure. The main feature that seems to characterize the group is their biosynthesis by a non-ribosomal mechanism, thus distinguishing the biosynthesis of these antibiotic peptides from that of general proteins. The amino acids are activated at the expense of ATP and the resultant aminoacyl adenylates react with sulfhydryl groups on a polyenzyme synthetase possessing a pantetheine residue. The activated aminoacyl groups become linked to the synthetase as thioesters and growth of the oligopeptide chain occurs on the pantetheinyl moiety, much as growing fatty acid chains are built upon fatty acid synthetases. Reports on bacitracin A,[61,62] gramicidin S,[63,64] polymyxins,[65] tyrocidine,[66] etamycin,[67,68] edeine,[69,70] and viomycin,[71,72] have appeared.



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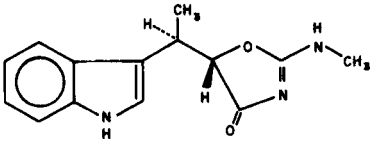
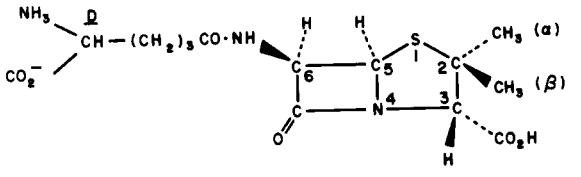


20

The phenoxazinone ring system of the *ACTINOMYCINS* (19) is apparently formed from kynurenine and 3-oxykynurenine with methylation taking place at a late stage [73]. Reports on amino acid variation and the mechanism of biosynthesis of the D-amino acids present in the side chains continue to appear [74,75,76,77,78].

INDOLMYCIN (20) is formed from pyruvate, and two enzymes active in initial stages of its biosynthesis have been studied. They are a transaminase and a C-methyltransferase. The hypothetical route to indolmycin is by indole pyruvate, 3-methyl-indolepyruvate, indolmycenic acid (reduced alpha oxo group) and finally indolmycin which probably takes its amidine group from an arginine molecule [79]. The closely related [pyrrolo (1,4) benzodiazepines] [80,81,82] antitumor antibiotics, anthramycin, tomaymycin and sibiromycin are formed from tryptophan (via the kynurenine pathway?), tyrosine and methionine-derived methyl groups [80,81,82].

The biosynthesis of the *BETA LACTAM ANTIBIOTICS* (penicillins or penams and cephalosporins or cephems) has been studied intensively. The synthesis of the basic systems by Cephalosporium species which make penicillin N (21) and cephalosporin C (22) seems to be from a linear tripeptide, delta L-α-amino-adipyl-L-cysteinyl-D-valine (23) [83]. Studies with specifically tritiated ^{14}C-labeled tripeptide and also with the same containing stereospecifically labeled valine (chiral methyl groups) have severely restricted the mechanistic possibilities for the cyclization steps. Among other observations, the valine methyl groups are incorporated without randomization [83, 84]. Presumably there is a common pathway in part for the formation both of the penam and cephem antibiotics. Model chemical reactions are at least compatible with the possibility that the beta lactam system is formed by either nucleophilic attack of amide nitrogen (from the valine) on a thioaldehyde (cysteinyl group) or alternately by oxidation of the same amide nitrogen followed by its nucleophilic displacement by an anion generated at the beta carbon of the cysteine residue [85]. It is clear that the D-configuration of the aminoadipyl side chain is generated at a late stage in the biosynthesis since the LLD tripeptide is a required precursor. Late stages in the biosynthesis of the cephem antibiotics also have been studied and seemingly deacetoxy cephalosporin C is formed first, followed by oxidation to desacetyl cephalosporin C and then acetylation to cephalosporin C[86].



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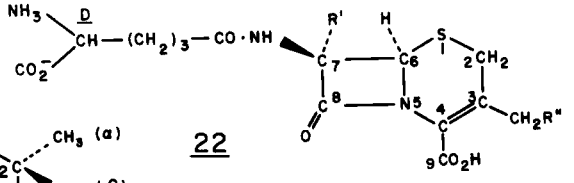


22

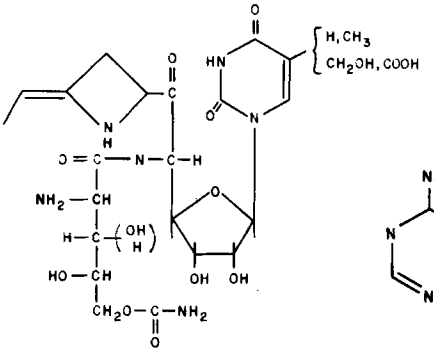
R' = H (OCH_3)

R" = H (Desacetoxy Ceph. C)

= OH (Desacetyl Ceph. C)

= OAc (Cephalosporin C)

Recent reports concerning biosynthesis of purine and pyrimidine-containing antibiotics deal with *PUROMYCIN* (an O-methyltransferase that promotes the last step in the biogenesis of puromycin by methylating the tyrosyl phenolic group) [87] and the *POLYOXINS* (24). The latter are an interesting family of antibiotics containing thymine or derivatives of it with oxidized methyl groups. A novel pathway for the biosynthesis of the thymine moiety has been proposed [88], one that is independent of the well-known thymidylate synthetase. The aldonic sugar residue is derived from L-glutamate [89] and the unique acid, 3-ethylidene-L-azetidien-2-carboxylic acid present in polyoxins A,F,H, and K comes from L-isoleucine [90]. Presumably carbamoyl phosphate is also required for the synthesis.



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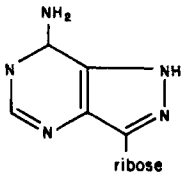

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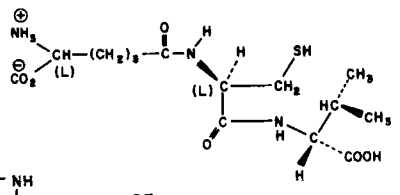

23

A series of papers on the biogenesis of the *FORMYCINS* (25) has appeared [91,92,93,94]. These pyrazolopyrimidine antitumor antibiotics are made from lysine (two carbons), glutamate, and ribose. At least three nitrogens including those in the pyrazole ring are derived from lysine.

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Chapter 15. Antiparasitic Agents

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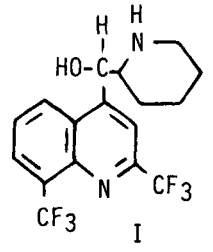
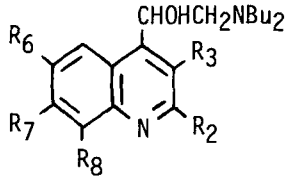
General - A compendium of chemotherapeutic agents for parasitic protozoa and helminths of dogs and cats was published.[1] Host-parasite relationships were reviewed.[2] The proceedings of an international conference on chemotherapeutic agents for parasitic diseases appeared.[3] Nucleoside analogs as antiparasitic agents were reviewed.[4]

Malaria - Reviews appeared on chemotherapy and prophylaxis[5] and drug resistance.[6] CDC malaria guidelines[7] were published. A test was described using *Plasmodium cynomolgi* in rhesus monkeys.[8] A method for predicting antimalarial activities of arylamidinoureas from their physicochemical properties was described.[9,10] Mefloquine (WR142,490) I was shown to be active against chloroquin resistant strains of *Plasmodium falciparum* in man for treatment[11] or prophylaxis[12] using single oral doses of 1 or 1.5 g.

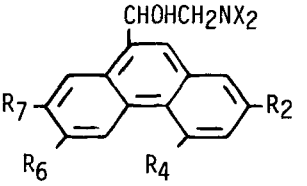


I

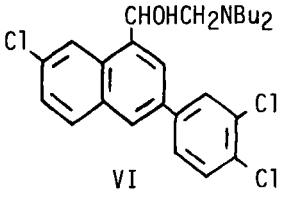
The synthesis of new quinoline methanols,[13,14] phenanthrene methanols,[15,16] and naphthalene methanols[17] continued, aimed at finding compounds of high activity and low phototoxicity. Against *Plasmodium berghei* in mice, II was active at 2.5 mg/kg and phototoxic at 100 mg/kg; III was the most active of the benzoquinolines but its phototoxicity was not determined. Among the phenanthrene methanols, IV was active at 20 mg/kg and V at 1.25 mg/kg. The naphthalene methanol VI was curative against *P. berghei* in mice at 10 mg/kg. Phototoxicity appeared to be absent from the series.



II R_2 = 4-ClPh; R_3 = F
R_6 = R_8 = Cl; R_7 = H
III R_2 = 2,4-Me_2Ph; R_3 = CH_3
R_6 = Cl; R_7R_8 = benzo



IV R_2 = R_4 = Cl; R_6 = SCH_3
R_7 = H; X = Bu
V R_2 = R_4 = CF_3; R_6 = R_7 = Cl
X = Pr



VI

The diastereomers of phenanthrene methanols and quinoline methanols showed striking differences in antimalarial activity,[18] postulated to relate to the distance between oxygen and the nonaromatic nitrogen atoms. No exciting new structures were described but activity was found in (4-oxo-2-oxazolin-2-yl)piperazines[19] and 1,2,4-triazines.[20]

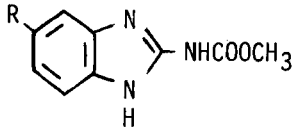
Ribosomes of *Plasmodium knowlesi* were isolated and characterized recently by Sherman *et al.*[21] These ribosomes sedimented in the 80S range and could be dissociated into 60S and 40S subparticles. The ribosomal RNA had a low % G+C of 37% and had sizes of 24.2S and 16.6S.[22] The ribosomes demonstrated high activity in poly(U)-directed synthesis of polyphenylalanine and were strongly inhibited by 10^{-4}M of nucleocidin, chlortetracycline, ethidium, puromycin, cycloheximide or berenil.[23] Similar studies have been also carried out on *Plasmodium lophurae*, and similar profile of drug sensitivities were demonstrated.[24] Most of the well-known antimalarial drugs tested showed no significant inhibitory activity in this *in vitro* assay.

However, clindamycin, a halogenated lincomycin analog, has been shown to be active against different species of malaria including several chloroquine-resistant strains of *P. falciparum*.[25,26,27] Further studies by Powers *et al.*[28] on *P. knowlesi* in rhesus monkeys indicated that clindamycin and its N-demethyl-4'-pentyl analog caused disintegration and disappearance of the parasite ribosomes. The phenomenon could be interpreted as the mode of action of the drug. Since lincomycin is neither effective clinically against malaria[28] nor active against eukaryotic microsomal ribosomes,[29] the activity of clindamycin suggests the possibility of uncovering drugs with a narrow spectrum of selectivity such that only protein synthesis by plasmodial ribosomes would be inhibited. Such an expectation has been buoyed by the activity against chloroquine-resistant malaria discovered in minocycline,[30,31] a derivative of tetracycline. Jacobs and Koontz[32] recently examined the rate of development of resistance to clindamycin or minocycline in *P. berghei* and found it much slower than to chloroquine, quinine or pyrimethamine. Clindamycin, doxymycin, tetracycline and spiramycin all showed causal prophylactic activity at the tissue stage of *P. berghei* development.[33]

The continuous cultivation of *P. falciparum* in human erythrocytes has been recently accomplished by Trager and Jensen.[34] The parasite, originally derived from an infected *Aotus trivirgatus* monkey, propagated over 100 million times by the addition of human erythrocytes at 3 to 4-day intervals. The parasite continued to reproduce asexually with a generation time of about 48 hours and remained infective to *Aotus*. This success may contribute to expanded efforts in immunological, chemotherapeutic and biochemical studies of malaria.

Helminthiasis - Reviews appeared on helminthiasis[35] and laboratory methods of screening for anthelmintics.[36] Two publications pointed out the resistance of field strains[37] and selected lines[38] of *Haemonchus contortus* to benzimidazoles, morantel and levamisole. A method for inducing *H. contortus* infections in the rabbit was described.[39] A new, highly potent benzimidazole, albendazole[40] VII, was reported to be active against nematodes, cestodes and trematodes. A single oral dose of 2.5 to 10 mg/kg in sheep and cattle eliminated 94-100% of these organisms. It was also effective in chickens, dogs and horses. Oxfendazole VIII was evaluated in sheep[41] and cattle[42] showing 92-100% activity against nematodes. Flubendazole IX was reported to be active against early and late encysted larvae of *Trichinella spiralis* in pigs.[43] Fenbendazole X was tested in sheep,[44,45,46] cattle,[47]

and pigs.[48,49] Broad activity against nematodes was reported in all cases. Activity in man against *Ascaris*, hookworm and *Trichuris* was reported.[50] Mebendazole XI was reported to be active against *Aspicularis tetraphera* and *Syphacia obvelata* infections in mice,[51] *Hymenolepis diminuta* in *Tribolium confusum*[52] and developing larvae of *Dirofilaria immitis* in dogs.[53] Oxibendazole XII was tested in cattle[54] and horses[55] showing 84-100% efficacy against mature parasites.



VII R = $SCH_2CH_2CH_3$

VIII R = $S(O)-C_6H_5$

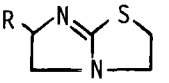
IX R = $C(O)-p-F-C_6H_4$

X R = $S-C_6H_5$

XI R = $C(O)-C_6H_5$

XII R = $OCH_2CH_2CH_3$

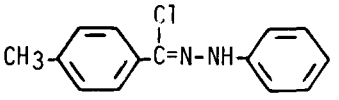
Newly described imidazothiazoles included nitramisole XIII[56] and butamisole XIV[57,58] which was 98-100% effective against *Trichuris vulpis* in dogs, orally or subcutaneously. Reduced activity was found for other worms.



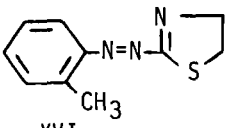
XIII R = $3-NO_2-C_6H_4$

XIV R = $3-(CH_3)_2CHCONH-C_6H_4$

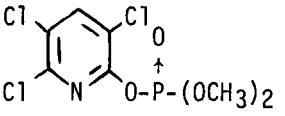
p-Toluoyl chloride phenylhydrazone (TCPH) XV was found to be effective against nematodes and cestodes of sheep.[59] A thiazoline XVI was reported to be orally effective against sheep nematodes.[60] Fospirate XVII eliminated *Echinococcus granulosus* and *Taenia hydatigena* infections from dogs.[61,62] Nitroscanate XVIII showed similar activity.[63,64] Other tapeworm compounds reported were praziquantel (Embay 8440) XIX effective in dogs and cats[65] and mebendazole effective in experimental infections[66] parenterally. Antibiotic S15-1 (SQ21,704), a member of the streptothricin family, eliminated tapeworms from cats, dogs and sheep.



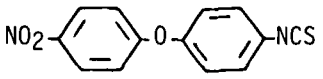
XV



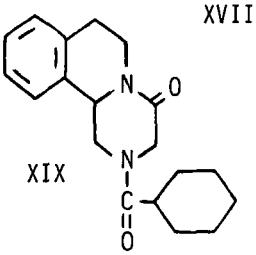
XVI



XVII

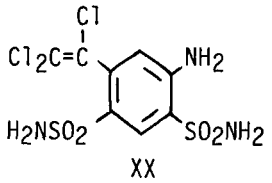


XVIII

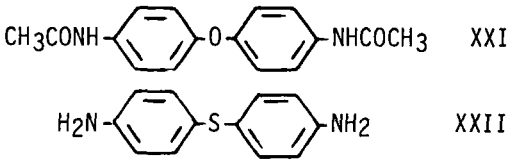


XIX

A novel disulfonamide XX was reported to eliminate mature and immature *Fasciola hepatica* from sheep and cattle at oral or subcutaneous doses of 2.5 to 30 mg/kg and showed no gross toxic symptoms up to 400 mg/kg in sheep.[68,69] Diamphenethide XXI was inactive against *F. hepatica* in the rat, presumably because it was not deacetylated to the free amine.[70] 4,4'-Diaminodiphenylsulfide XXII and its diacetyl derivative were 62-100% effective against 3-week-old *F. hepatica* infections in sheep.[71]



XX



XXI

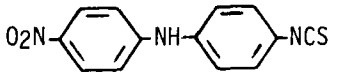
XXII

The effect of *in vivo* treatment with mebendazole XI on the intestinal ultrastructures of *Ascaris suum* and *Syngamus trachea* was studied.[72,73] Six hours after the onset of medication, microtubules disappeared from the apical cytoplasm of intestinal cells in both parasites. No change in distribution and number of microtubules in the cytoplasm of intestinal epithelial cells was observed among host animals under the same drug treatment up to at least 24 hours. However, a very close analog of mebendazole, oncodazole [R17934, methyl-5-(2-thienylcarbonyl)benzimidazole-2-carbamate] has been presented recently as an antitumor agent.[74] The drug was capable of arresting mitosis at metaphase of L1210 leukemia ascites cells *in vivo*,[75] and interfering with microtubules in mammalian cells cultured *in vitro*.[76] Further studies indicated that oncodazole binds to rat brain tubulin in a mole to mole ratio and inhibits the polymerization of tubulin and the binding of colchicine to tubulin.[77] It was also more active than colchicine in promoting concanavalin A capping on human polymorphonuclear leukocytes.[78]

Although it is not known whether mebendazole has antitumor activities or oncodazole possesses anthelmintic activities, it is not unlikely that each drug may demonstrate both activities. It is also possible that the mechanism of anthelmintic activity by mebendazole could be primarily by its anti-microtubule action. Since cross-resistance between mebendazole and other benzimidazole anthelmintics has been repeatedly demonstrated in *H. contortus* in laboratory as well as in natural environments,[79] it is tempting to assume that all the benzimidazoles act by inhibiting formation of microtubules in the parasites. This hypothesis is being favored by the authors over the other theory that the mode of action of some benzimidazoles may be by inhibiting fumarate reductase[80] because of several reasons: 1) ID_{50} of the inhibition of tubulin polymerization by oncodazole is 0.63×10^{-6}M whereas ID_{50} of the inhibition of fumarate reductase by thiabendazole is about 1×10^{-3}M.[81] 2) Thiabendazole is much more effective in inhibiting hatching of the parasitic nematodes[82] than its inhibition of fumarate reductase. 3) The correlation between *in vivo* resistance and resistance of fumarate reductase to thiabendazole or cambendazole has been poor.[81] Final proof or disproof will have to wait for further studies.

Schistosomiasis - In a series of reviews, clinically available antischistosomal drugs,[83] screening procedures,[84] and the mutagenic evaluation of antischistosomal drugs[85,86,87,88,89,90] were discussed. The experimental chemotherapy, pharmacology and toxicology of hycanthone was reviewed.[91] Studies on juvenile and adult schistosomes *in vitro* indicated that this type of evaluation was not useful as a screening technique for new antischistosomal drugs.[92] 4-Isothiocyanato-4'-nitrodiphenylamine (C9333-GO/CGP4540) XXIII was found to display an unusual spectrum of anthelmintic activity against *Nematospiroides dubius* in mice and *Schistosoma haemotobium, Schistosoma*

mansoni and *Schistosoma japonicum* in various hosts including mice, jirds and primates.[93] The drug was effective by oral or parenteral administration in single doses of 5 to 120 mg/kg and well tolerated in acute toxicity tests. Its effectiveness was found to vary with particle size of the drug and the age of the parasite.[94] It was suggested that a mutagenic metabolite of XXIII was formed by intestinal bacteria.[94] A group of trisubstituted aminoethane thiols were active *vs. S. mansoni* in mice.[95] The most active was XXIV.



XXIII

$Me_3CNHCH_2CH_2SH$

XXIV

Trypanosomiasis - Reviews were published on the chemotherapy of African trypanosomiasis[96] and *Trypanosoma cruzi* infections.[97] A test system was developed using *Trypanosoma rhodesiense* in mice, suitable for screening large numbers of compounds.[98] Some novel nitrofurfuryl hydrazones were active against *T. cruzi* in mice.[99] Differential screening revealed nine compounds active against *T. cruzi* in cell culture which were non-toxic to African green monkey kidney cells.[100]

The bloodstream forms of African trypanosomes depend on glycolysis (from glucose to pyruvate) for energy supply because a mitochondrial respiratory chain is lacking.[101,102] NADH generated by glyceraldehyde phosphate dehydrogenase is reoxidized by an α-glycerol phosphate oxidase system unique to trypanosomes.[103,104] It has long been postulated that this oxidase system should provide an ideal target for a selective chemotherapeutic agent.[103,104] Some aromatic hydroxamic acids have been shown to inhibit O_2 uptake of bloodstream trypanosomes at about 10^{-4}M.[105,106] One of these compounds, salicylhydroxamic acid (SHAM), was shown to block the oxidase part of the system with a Ki value of 2.5 μM.[107] However, no therapeutic effect was observed on *Trypanosoma brucei* when SHAM was tested *in vivo*[108] up to a plasma concentration of 10^{-4}M for 4 hours which suggested that O_2 uptake may not be essential for the survival of trypanosomes in the bloodstream. Similar results were obtained *in vitro* when *T. brucei*, supplied with glucose, survived for hours after O_2 uptake had been blocked by SHAM. These findings defy the generally accepted glycolytic pathway for the parasite which predicts no net gain of ATP under anaerobic conditions.[102] The scheme of metabolism thus must have been wrong.[107]

Although the correct pathway of anaerobic glucose metabolism in African trypanosomes still remains unknown, the production of equimolar amounts of pyruvate and glycerol under anaerobic conditions has been repeatedly demonstrated.[107,109,110] Clarkson and Brohn[111] were able to utilize these facts to observe immediate loss of motility of *T. brucei in vitro* in the presence of 1mM SHAM and 2.5mM glycerol. Intravenous injection of *T. brucei* infected rats with SHAM (96 mg/kg) and glycerol (276 mg/kg) immobilized all the parasites within 1 minute and no intact motile parasites were found after 3 minutes. Similar effects by the SHAM-glycerol combination were also observed on *T. rhodesiense*. However, the recurrence of parasitemia always took place following the treatment.

Damper and Patton[112] studied transport of pentamidine in *T. brucei*

and identified the presence of a highly specific pentamidine transport system in the blood trypomastigote form. The transport showed saturation kinetics and an average Km value of 2.68 μM. Stilbamidine, propamidine, hydroxystilbamidine and benzamidine are all competitively inhibitory with K_I values of 3.5, 3.3, 1.7 and 6.5 μM respectively. SHAM (5mM) and iodoacetate (1mM) inhibited the transport activity by 78% and 47% suggesting its possible dependence on energy supplied by aerobic metabolism. The kinetics of uptake was altered in dyskinetoplastic strains of *T. brucei* which are resistant to pentamidine.[113] They showed lower rates and higher Km for pentamidine transport as did *T. rhodesiense* which is characteristically less sensitive to the drug. This alteration in uptake may provide basis for pentamidine resistance and cross-resistance among aromatic amidines.

Leishmaniasis - Over 100 compounds with known antiparasitic activity were tested against *Leishmania mexicana mexicana* M379, *Leishmania tropica major P*, and *Leishmania donovani* HV3 in cell culture.[114] Several antimalarial compounds including 5-, 6- and 8-aminoquinolines, quinazolines, amidinoureas and dihydrofolate reductase inhibitors showed sufficient activity to warrant further study.

Trichomoniasis - A review of new antitrichomonal agents appeared.[115] An experimental rat model system was described[116] using a dual infection of *Candida albicans* and *Trichomonas vaginalis* as a screening method. A soluble complex of the polyene antibiotic mepartricin and sodium lauryl sulfate was reported to be orally effective in patients with vaginal trichomoniasis.[117] A series of phosphorylated derivatives of carboxylic acids[118] was found to have trichomonocidal activity.

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole], a well-known agent against *T. vaginalis* infection,[119] inhibits anaerobic protozoa and anaerobic bacteria in general but has limited activity against aerobic organisms.[120] As the mechanism of its action, it has been suggested that an intermediate in the reduction of metronidazole, produced only in anaerobes, is bound to DNA and protein and inhibits subsequent nucleic acid synthesis.[121] The hypothesis gained some support from the findings that extracts of trichomonads and anaerobic bacteria reduce the drug[122,123] and that O_2 markedly suppresses the uptake of the drug in trichomonads and *Entamoeba invadens*,[124] even though the reduced product of metronidazole has not yet been characterized.[121] Recent studies,[125,126,127] however, seem to cast some doubt on the possible binding of the reduced drug to DNA. Ings and Constable found that ^{14}C-metronidazole did not accumulate at any particular site within *T. vaginalis* but was evenly distributed in the nucleus and cytoplasm.[125] Though there is some disagreement on whether there was any change about the nuclei of drug-treated *T. vaginalis*,[125,126,127] at least two investigators appeared to agree that the number of polyribosomes had decreased whereas the number of single ribosomes in the cytoplasm had increased as a result of drug treatment.[125,126] Metronidazole thus may impair protein synthesis in *T. vaginalis*.

Nitrofurans, such as nitrofurazone, nitrofurantoin and SQ 18506, have

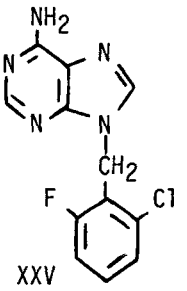
long been used as urinary tract antiseptics.[128] They have been recently identified as agents also active *in vitro* against *T. vaginalis*.[127,129] Buchner and Edwards[127] indicated that the morphological changes in *T. vaginalis* caused by nitrofurans were very similar to those by metronidazole. McCalla, Reuvers and Kaiser[130,131] demonstrated that the antimicrobial activity of nitrofurans was linked with reduction of the nitro group and the reduced nitrofurans in turn inhibit DNA function and cause chromosome breakage in bacteria; a mechanism very similar to that postulated for metronidazole though not restricted among anaerobes. However, another bacteriostatic activity of nitrofurantoin at much lower doses (0.5-25 μg/ml) has been noted recently by Herrlich and Schweiger.[132] They found in *Escherichia coli* no effect by nitrofurans on transcription, but the drugs inhibit specifically the expression of inducible genes, *e.g.*, β-galactosidase, tryptophanase, galactokinase, *etc.* Similar activity was expressed in the *E. coli* protein synthesis assay *in vitro*, and the inhibition of messenger RNA expression occurred at the initiation step, suggesting selective translational control.

All the evidence reviewed above points to an interesting possibility that the primary action of metronidazole and nitrofurans in *T. vaginalis* may be the inhibition of synthesis of proteins. Whether reduction of the nitro group is the prerequisite for this activity remains to be seen.

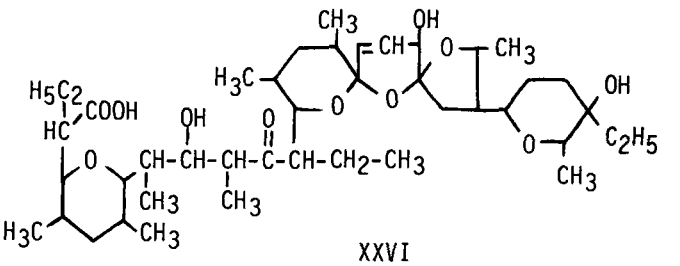
Coccidiosis - Reviews appeared on coccidiosis of sheep[133] and propylactic immunization.[134] In a study using recent field isolates, no anticoccidial was effective against all isolates.[135] Sequential use of amprolium, nicarbazin, unistat and zoalene against *Eimeria tenella* produced a strain sensitive to nicarbazin but resistant to the other agents.[136] A new anticoccidial MK-302 XXV was reported to control *Eimeria acervulina, Eimeria brunetti, Eimeria maxima, Eimeria necatrix,* and *E. tenella*.[137,138] New anticoccidial ionophores described included narasin (A-28086) XXVI,[139] with predominantly Na^+ selectivity[140] and CP-39,295[141] XXVII. Monensin was reported to be active in sheep[142] and to have profound effects on rumen microbiology.[143,144]

Further studies on pyridoxal analogs showed XXVIII[145] and XXIX[146] to have activity against *E. acervulina*. A series of pyran-3(4H)-ones, exemplified by XXX, were active against *E. acervulina* and *E. tenella*.[147]

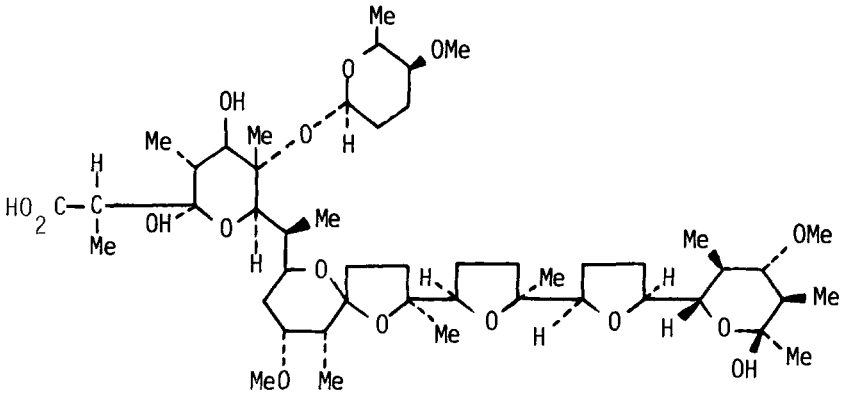
The mechanism of action of quinolone coccidiostats was recently studied by Wang in *E. tenella*.[148,149] Amquinate, buquinolate, methyl benzoquate and decoquinate were all reversible inhibitors of *E. tenella* respiration as well as sporulation. The ID_{50} values were 1 to 2x 10^{-5}M against respiration during sporulation and 3 x 10^{-6}M against respiration during excystation. Respiration in isolated *E. tenella* mitochondria was inhibited 50% by the quinolones at 3 pmoles per mg protein at the site near cytochrome b. Mitochondria of an *E. tenella* amquinate-resistant mutant were at least 100-fold less susceptible to the quinolones, while chicken liver mitochondria showed no sensitivity to the drugs. It was thus suggested that the quinolones may suppress the parasites by inhibiting their mitochondrial respiration. It was also postulated that the high frequency of resistance



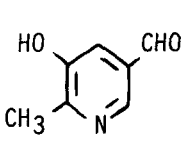
XXV



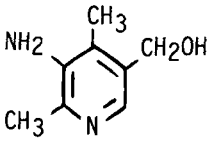
XXVI



XXVII



XXVIII



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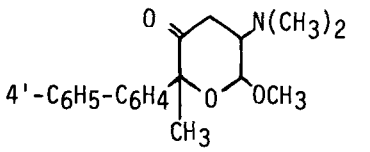

XXX

to the quinolones among coccidia[150,151] could be due to autonomous genetic control of drug-resistance in mitochondria.

The phenomenon of drug resistance has been studied by Joyner and Norton.[152,153] They found that when two drug-resistant strains of *E. maxima* were passaged together in untreated chickens, at least some of the resultant oocysts became resistant to both drugs. Acquisition of resistance by one strain from the other occurred between methyl benzoquate and sulfaquinoxaline or clopidol and sulfaquinoxaline but not between clopidol and methyl benzoquate. The transfer of drug resistance did not take place between different species but occurred readily between different variants of the same species 96 hours after inoculation or later.[154] Treatment with acriflavin had no effect on the transfer suggesting genetic recombination.

Dihydrofolate reductase was found and purified from unsporulated

oocysts of *E. tenella* by Wang, *et al.*[155] Its molecular weight was estimated as 240,000 daltons and it was strongly inhibited by pyrimethamine (K_I = 3nM).

Cultivation of *E. tenella* in cell cultures has been widely utilized in studying known anticoccidials and provided useful information on these compounds.[156,157] The technique was recently further developed for the primary screening of compounds in several laboratories.[158,159] In a recent report,[160] however, Ryley summarized the results from screening of 11,550 compounds in cell cultures and concluded that the screening method is not a satisfactory or reliable alternative to screening in chickens.

Toxoplasmosis - Using *Toxoplasma gondii* in cell culture,[161] the polyether ionophores lasalocid and monensin were highly active whereas ormetoprim and sulfadimethoxime or a combination were inactive or weakly active.

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Section IV - Metabolic Diseases and Endocrine Function

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Chapter 16. Cellular Responses Mediating Chronic Inflammatory Diseases

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Introduction - The etiology of chronic inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, and sarcoidosis remains unknown. However, a chronic inflammatory component is recognized to occur in these diseases because of an influx of blood leukocytes and a proliferation of local cells. An example of this occurs in diarthrodial joints involved in rheumatoid arthritis[1,2] where synovial lining cells proliferate, overlying a mass of fibrous tissue, termed pannus, which is infiltrated by numerous lymphocytes, macrophages, and fibroblasts. The synovial fluid of such joints is increased in volume and contains large numbers of polymorphonuclear leukocytes (PMN), lymphocytes, and macrophages. In addition to the cellular components present at sites of inflammation, the products of various humoral systems associated with inflammatory events accumulate--viz, fibrin, cleavage products of the complement system, kinins, and antigen-antibody complexes. We will restrict our remarks to some cellular aspects of chronic inflammatory processes, giving particular attention to biochemical changes occurring in cells responding to inflammatory stimuli. These changes often lead to the secretion of products which modulate inflammatory processes in various ways. Some inflammatory stimuli interact directly with phagocytic cells, causing the release of their mediators. For example, urate crystals release mediators of gouty inflammation from PMN, while the interaction of toxic materials such as asbestos, silica, and thermophilic yeasts with alveolar phagocytes leads to chronic inflammatory changes in the lung. These are nonimmunologically mediated inflammatory events, and there is no evidence for a primary involvement of lymphocytes in this type of inflammation. More commonly, chronic inflammatory processes are the result of the interaction of a specific immunogenic stimulus with lymphocytes. Further exposure of sensitized lymphocytes to the specific immunogen results in the synthesis and release of inflammatory mediators. These products of stimulated lymphocytes act directly upon target tissues, e.g., lymphotoxin, or alternatively influence the activity of other cells, e.g., migration inhibitory factor (MIF), acting upon macrophages.

In order to develop meaningful assay systems to study mechanisms involved in chronic inflammation, cells from cartilage, bone, normal synovium, and from organized inflammatory lesions as well as leukocytes from spleen, thymus, and macrophages from serous cavities of laboratory animals maintained as stable populations in cell or organ culture have been utilized.

Recent improvements in methodology for cell isolation from normal and inflamed tissues have permitted the maintenance of cell culture systems

relevant to *in vivo* situations. This has been achieved by dispersing tissues by the judicious use of connective tissue degrading enzymes rather than mechanical disaggregation or mincing of tissue and incubation with trypsin. Clearly, the complex cellular interactions underlying chronic inflammation are best studied using methods of cell and organ culture where the responses of individual cell types to various stimuli can be defined. While such systems fail to provide a comprehensive model for any chronic inflammatory disease, they do provide definitive information on the response of a given cell type to stimuli introduced into its environment. Much of the information reviewed in this chapter has been derived in this way. Only a judicious combination of these test systems will provide comprehensive assays for the various aspects of inflammatory disease. An extensive background to this brief survey is found in the proceedings of two recent international meetings on inflammation.[3,4]

Chemotaxis - Chemotaxis is the mechanism by which cells are attracted to sites of inflammation. Leukocytes move in a specific direction established by a concentration gradient of a stimulus generated at a site of inflammation. The Boyden chamber[5] still serves as the basic apparatus for measuring chemotaxis, although improvements have been described[6] and a method using radiolabeled cells is in use.[7] An excellent monograph on the subject of chemotaxis has appeared recently.[8] Activation of the complement systems leads to formation of several molecules with chemotactic activity, including C3a, C5a, and the trimolecular complex C567. Human C3a has been purified, sequenced, and shown to be a cationic peptide containing 77 amino acid residues with a molecular weight of 9,000.[9] Human C5a contains 73 amino acid residues with additional carbohydrate accounting for 25% of its molecular weight of approximately 11,000 daltons.[10]

The eosinophil chemotactic factor of anaphylaxis (ECF-A) is a preformed product found in rat mast cell granules,[11] in human lung, nasal polyps, and PMN.[12] The activity resides in two acidic tetrapeptides,[13] Ala-Glu-Ser-Glu and Val-Gly-Ser-Glu, which preferentially attract eosinophils.[14] Synthetic peptides of this composition are equipotent to natural ECF-A both *in vitro* and *in vivo*.[15] ECF-A shows maximal chemotactic activity at 10^{-9} M, and concentrations as low as 10^{-13} M deactivate eosinophils to subsequent stimulation by chemotactic concentrations of ECF-A and C5a.[16,17] The COOH terminal tripeptide of ECF-A, Gly-Ser-Glu, is only weakly chemotactic but it causes dose-dependent suppression of eosinophil chemotactic responses to Val-Gly-Ser-Glu.[16,17]

Simple formyl methionyl peptides stimulate leukocyte movement and are chemotactic.[18] Becker *et al*[19,20] have synthesized a series of di-, tri-, and tetrapeptides, most of them being formyl methionyl peptides. These are chemotactic, stimulate phagocytosis of latex particles, and cause the selective release of lysosomal enzymes from PMN in the presence of cytochalasin B. There is a high degree of correlation between the ability of the peptides to enhance PMN movement and cause selective release of acid hydrolases. The presence of the formyl group on the methionine leads to a 3,000- to 30,000-fold increase in chemotactic activity. Further studies

will determine if these oligopeptides bind to a specific receptor and whether such a receptor is involved in initiating basic functions of the PMN, such as chemotaxis and phagocytosis. Hook et al[21] have shown that formyl methionyl peptides trigger the release of histamine from basophils.

Arachidonic acid released from phospholipids or neutral lipids at sites of inflammation can be converted via the cyclooxygenase system into the biologically active endoperoxide prostaglandin G_2 as well as several non-prostanoate hydroxy fatty acids.[22] Alternatively, it may be converted by a lipoxygenase into 2-L-hydroxy-5,8,10-14-eicosatetraneoic acid (HETE).[23] HETE produced by platelet aggregation is chemotactic for PMN (see Chapter 19).

Cellular Mediators of Inflammatory Processes

Lymphocyte Products - It is only 10 years since it was realized that lymphocytes are heterogenous[24] in terms of their function and that cell-to-cell collaboration between lymphocyte subclasses is essential for proper immune function.[25] Subsequently it has become clear that several subsets of lymphocytes recognized by their surface antigens[26,27] mediate specific functions.

Lymphocytes responding to antigens or mitogens synthesize and secrete macromolecular products termed lymphokines that possess a variety of biological activities[28,29] which are thought to initiate many of the changes seen in delayed hypersensitivity responses. Extreme difficulty has been encountered in purifying and characterizing these macromolecules.[30] Some progress has been reported toward the preparation of an antiserum with specificity toward lymphokines that influence macrophage functions,[31] and also toward products participating in mixed lymphocyte responses.[32] Antibodies prepared against guinea pig lymphotoxin did not neutralize mitogenic factor or MIF.[33] Mouse lymphotoxin has been characterized as a molecule of 41,000 daltons with an isoelectric point of 4.4 to 4.8.[34] Human tonsillar lymphocytes produce a lymphotoxin of molecular weight of approximately 80,000, while human peripheral blood leukocytes produce a lymphotoxin with a molecular weight of approximately 45,000.[35]

MIF inhibits the amoeboid movement of macrophages. This material has been partially purified from human,[36] guinea pig,[37] and murine[38] sources. Its receptor on macrophages is susceptible to inactivation by α-fucosidase.[39]

Suppressor lymphocytes[40] control immune responses in part by secretion of an antigen-specific factor which is not active across histocompatibility barriers.[41] This product has a molecular weight between 35,000 and 55,000 daltons and can be completely absorbed with an alloantiserum specific for the I region of the major histocompatibility complex.[42] A soluble immune response suppressor (SIRS) is produced by concanavalin A-stimulated murine splenic lymphocytes[43] and inhibits plaque-forming responses of B lymphocytes and cytotoxic lymphocyte responses[44] to alloantigens. SIRS has a molecular weight between 48,000 and 67,000,[44] does

not contain any immunoglobulin-like material, and is destroyed by proteinases and nucleases. Its inhibitory activity may be mediated by interference with the accessory role played by macrophages in the response of these cells.[43]

Macrophage Products - Mononuclear phagocytes maintained in tissue culture synthesize and secrete a number of products relevant to chronic inflammatory processes.[45] In some instances these are secreted in direct response to inflammatory stimuli.[45] Neutral proteinase secretion is stimulated by intraperitoneal injection of thioglycollate broth, which acts as a sterile inflammatory stimulus.[46] Cells harvested in this manner secrete plasminogen activator,[47] elastase,[48] and collagenase.[49] Collagenase secretion by mineral oil-induced guinea pig peritoneal macrophages is stimulated by the addition of supernatants of antigen- or concanavalin A-stimulated guinea pig lymphocytes[50] or directly by bacterial lipopolysaccharides.[51] Collagenase is secreted by alveolar macrophages from normal rabbits[52,53] or animals sensitized with Freund's complete adjuvant,[52] larger amounts of enzyme being secreted from the latter. Alveolar macrophages secrete collagenase in a latent form under certain conditions,[52] but they also secrete a proteinase which activates the latent enzyme under physiological conditions.[52] Macrophages harvested from mice given an intraperitoneal injection of chrysotile asbestos, a potent stimulus of chronic inflammation, secrete plasminogen activator; but mice injected with latex particles, which have little inflammatory activity, do not secrete this enzyme.[54] Neutral proteinase secretion by macrophages is dependent upon an intact protein synthetic mechanism.[49] Glucocorticoids inhibit secretion of plasminogen activator at doses close to physiological levels and corresponding to their anti-inflammatory potencies.[55] Colchicine and vinblastine inhibit plasminogen activator secretion at 1×10^{-7} M. Cholera toxin is an extremely potent inhibitor of secretion, reducing release by 90% at 10^{-12} M.[55] Nonsteroidal anti-inflammatory drugs have no detectable effects on secretion of plasminogen activator. In contrast to its inhibition of plasminogen activator release by macrophages, colchicine stimulates the secretion of elastase, collagenase, and a neutral proteinase hydrolyzing azocasein by these cells.[56]

Acid hydrolase release from macrophages is not a general response to phagocytic stimuli, as is the case with PMN. Enzyme release by macrophages occurs only with inflammatory stimuli. Stimuli causing lysosomal hydrolase release may be those causing nonimmune-based inflammation, such as Group A streptococcal cell walls, carrageenan, zymosan, asbestos, or products of lymphocyte stimulation such as lymphokines and antigen-antibody complexes.[45] In addition, products of the activation of the complement system, C3b in particular, induce selective release of acid hydrolases from macrophages.[57,58] This observation is of particular interest since macrophages synthesize those factors of the alternative pathway of complement required for the conversion of C3 to C3a and C3b.[59,60] Since several inflammatory stimuli, e.g, bacterial lipopolysaccharides, carrageenan, and zymosan, are known to activate the alternate pathway of complement, the formation of C3b may be an intermediate step for the expression of macrophage functions relevant to its role in inflam-

matory processes.[61] The selective release of acid hydrolases is inhibited by anti-inflammatory drugs under certain conditions. Glucocorticoids inhibit acid hydrolase release from thioglycollate-induced macrophages caused by zymosan.[62] Preincubation with indomethacin inhibits the release of acid hydrolases caused by Group A streptococcal cell walls.[63]

Macrophages secrete products which stimulate both the proliferation of fibroblasts and their synthesis of collagen.[65]

Macrophages secrete prostaglandins in response to several types of inflammatory stimuli. Oil-induced guinea pig peritoneal macrophages secrete PGE_2 and $PGF_{2\alpha}$ when cultured in the presence of lymphokines.[66] Both thioglycollate-induced and unstimulated mouse macrophages secrete prostaglandins in response to an inflammatory stimulus such as zymosan but not to a stimulus lacking inflammatory capacity, namely, latex particles.[67]

Macrophages play an important role in the initiation and modulation of lymphocyte responses to antigen.[68] They secrete factors[69] which enhance the response of both T lymphocytes[70] and B lymphocytes[71,72] to mitogenic stimuli.

PMN Products - Neutral proteinases of PMN lysosomes[73] degrade a wide variety of connective tissue substrates including elastin, proteoglycan, and collagen.[74] Cathepsin G has been found only in PMN, and elastase from these cells has a substrate specificity different from that of pancreatic and macrophage elastase.[75] In addition to their effects on connective tissue components, the neutral proteinases of PMN lysosomes also stimulate the activity of other cells involved in the inflammatory response. Both elastase and cathepsin G from human PMN stimulate the incorporation of thymidine by human peripheral blood and splenic lymphocytes.[76] The stimulated lymphocytes are of the B lineage, with no effect seen on T lymphocytes.

Basophil Products - Basophilic leukocytes participate in both delayed, cell-mediated, and immediate, homocytotropic antibody-induced hypersensitivity reactions. Basophils contain several pharmacological mediators that are released in response to inflammatory stimuli.[77] These mediators include histamine, slow-reacting substance of anaphylaxis (SRS-A), ECF-A, and platelet activating factor (PAF). The degranulation process has been observed morphologically in allergic contact dermatitis in man.[78] C5a causes the release of histamine from human basophils.[79,80] The C5a-induced release is dependent upon calcium ions and occurs within 2 minutes of C5a addition, it is additive to that induced by IgE, and there is no cross-desensitization between the two stimuli of release.[81,82] Short-term cultures of human[83] and guinea pig basophils[84] have been established. Basophils from the latter species were shown to synthesize histamine,[84] whereas human basophils do not.[83]

PAF is secreted from basophils by an IgE-dependent reaction, has a molecular weight of approximately 300, and is sensitive to phospholipase D.[85] It causes the secretion of vasoactive amines from platelets by a calcium-

requiring and temperature-dependent process[86] which is inhibited by serine proteinase inhibitors and agents elevating cellular levels of cyclic AMP.[87]

Production of Mediators in Response to Inflammatory Stimuli by Organized Tissues Maintained in Culture

Organ Culture of Synovium - Organ culture is defined as the cultivation *in vitro* of tissues in a differentiated, functional state similar to that of their organs of derivation.[88] Fell *et al*[89] have used organ culture techniques to study the physiology and pathology of synovium and cartilage. Cartilage devoid of soft connective tissue responds to complement-sufficient antiserum with resorption of its matrix. The proteoglycan component, but not collagen,[90] is degraded. When synovial soft connective tissue is added to the cartilage system, both connective tissue components are degraded.[91] Therefore, cultures of the components of connective tissue can serve as models for the study of proteoglycan and collagen breakdown and the effects of various agents on such processes.

Dispersed Cell Culture of Synovial Tissue - Although organ culture studies yield information regarding mechanisms involved in joint and rheumatoid diseases, they do not allow the determination of which cell type or types are responsible for the observed effects. Organ cultures of human rheumatoid synovium secrete prostaglandins[92] and certain neutral proteinases.[93] Dayer *et al*,[94] using crude collagenase followed by trypsin treatment, have dispersed human rheumatoid synovium and initiated cultures containing heterogeneous populations of cells as judged by morphological criteria.[94] These cells produce collagenase and prostaglandins, and the synthesis of these products correlated with the number of cells which were adherent, had Fc receptors, and secreted lysozyme.[94] Collagenase production by the cultured cells is stimulated by a human lymphocyte factor.[95]

Cultured cells from enzymatically dispersed, carrageenan-induced granulomas display several characteristics of mononuclear phagocytes--namely, Fc receptors, phagocytic activity, and the constitutive secretion of lysozyme.[96]

Acute Phase Protein Production by Liver - The liver synthesizes a number of acute phase proteins in response to ill-defined products of chronic inflammatory lesions. These proteins include haptoglobin, ceruloplasmin, α-1 trypsin inhibitor, C-reactive protein, and fibrinogen.[97-99] Identification of the cell type in the liver that is responsible for the synthesis of these proteins remains to be established. The process of acute phase protein synthesis has been examined by two general protocols, (1) liver perfusion and (2) incubation of liver slices.

1. Liver Perfusion - Normal intact rat liver can be successfully perfused for 12 hours,[100] and John and Miller[100,101] have demonstrated net biosynthesis of albumin, fibrinogen, α-1 acid glycoprotein, and α-2 (acute phase) globulin. Synthesis of the acute phase proteins can be induced by perfusion medium supplemented with insulin, cortisol, growth hormone, and

amino acids.[100]

2. Liver Slices - Liver slices prepared from rats bearing an inflammatory lesion caused by subcutaneous injection of turpentine show an increased capacity for the incorporation of radioactive amino acids and glucosamine into acute phase proteins.[102]

A third approach to study the synthesis of acute phase proteins is to use primary cultures of homogenous preparations of adult liver parenchymal cells which can be maintained in culture without division up to 1 week.[103,104] Employing such a system, the synthesis and secretion of several serum proteins such as albumin, fibrinogen, transferrin, and α-1 acid glycoprotein has been demonstrated.[103,105]

In addition, it is now possible to isolate parenchymal and Kupffer cells from the same liver.[106] Therefore, isolation and cultivation of these two cell types should allow the determination of the cellular origin of the acute phase proteins as well as the products of inflammatory processes which stimulate their production.

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Chapter 17. Molecular Mechanisms and Pharmacological Modulation in Psoriasis

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Introduction - Psoriasis is a skin disease which occurs in separated patches over the body. Its causes are probably multiple genetic and environmental factors acting in concert.[1] Of these factors, the three- to fourfold increased frequency of the cell surface gene products HL-A13 and HL-A17[2] and induction of psoriasis by cutaneous trauma[3] are well established. However, these established facts do not as yet immediately suggest areas of research with therapeutic application. Therefore, we have been concerned with those aspects of the molecular pathophysiology of psoriasis which are hypothetically amenable to pharmacological modulation. When using this approach, research is not restricted by not knowing which molecular events are primary versus secondary. That is not to say that knowledge of primary versus secondary molecular events is unimportant. Quite the contrary, such knowledge might be essential to successful therapeutics, but such studies at the molecular level are not generally feasible at this time in normal or diseased human skin. An important corollary of this research strategy is that in initiation and/or maintenance of a lesion certain but not all molecular events, whether they are primary or secondary, are critical. We have arbitrarily designated these events as "critical molecular events in pathophysiology". The postulate is that without these events, the lesion would either not develop or would spontaneously disappear.

We have attempted to establish what certain of these "critical molecular events in pathophysiology" might be by examining lesional epidermis of psoriasis and comparing it to uninvolved psoriatic epidermis and to epidermis of normal subjects. Much psoriasis research has focused on the epidermis for several reasons. Although the clinical lesion of psoriasis has several compartments (i.e., dermal, vascular, inflammatory, immunologic, nervous, etc.) the epidermal compartment is the only one which can be surgically obtained in sufficient quantity, with reasonable homogeneity, and with minimal scarring of the patient. Epidermal research in psoriasis is reasonable and justifiable because without abnormal epidermal homeostasis, the psoriatic lesion could not exist, or at least would not pose a significant clinical problem. The fact that most, if not all, psoriatic lesions sooner or later spontaneously disappear indicates that certain "critical molecular events" occur spontaneously within the epidermis to either promote or permit the lesion to revert to normal. Such "critical molecular events in pathophysiology" are viewed as ideal candidates for pharmacological modulation. When possible, the pharmacological agents employed should be those which exist naturally in human skin, i.e., the concept of orthomolecular pharmacology. It seems rational to suggest that in principle, the manipulation of naturally occurring molecules will be a safer approach than the utilization of classical anticancer drugs or agents which are potential mutagens. Those events in the epidermal pathophysiology of psoriasis which we consider critical will be reviewed after which

their pharmacological manipulation will be discussed.

Cell Surface in Psoriasis - The first of these factors which we believe are "critical" is the cell surface. In most, if not all animal cells, the cell surface appears to participate in the regulation of proliferation and tissue cytodifferentiation.[4] In psoriasis the lesional epidermal cell surface is markedly reduced by ultrastructural analysis.[5,6] This may be a general feature of a stimulated epidermis, thus being nonspecific for psoriasis. Since normal epidermal growth regulation may be impossible without a normal cell surface,[5,6] such potential nonspecificity should not diminish the importance of this observation. As mentioned above, HL-A13 and HL-A17 gene products are cell surface constituents which have a three- to fourfold elevated frequency in psoriasis.[2] These HL-A abnormalities may participate in the development of a psoriatic lesion and not be merely genetic markers. Several observations suggest this possibility.

The H-2 gene complex in the mouse is analogous to the HL-A gene complex in man. In mice a particular H-2 genotype is one regulator of the level of cellular cyclic AMP.[7] The cell surface membrane H-2 phenotype presumably is controlled by a specific H-2 genotype. By analogy, the HL-A genotype in man may do likewise. The abnormal HL-A psoriatic phenotype could alter cell adhesion-mediated growth control in the epidermis. The abnormal HL-A phenotype could alternatively interfere with potential physiological regulation of epidermal cyclic AMP content by endogenous beta catecholamine. We have previously shown that exogenously added beta catecholamine elevates the epidermal cyclic AMP content, an elevation which is blocked by propranolol.[8] This is especially interesting in light of the suggested common evolutionary origin of HL-A antigens and beta receptors.[9] Furthermore, Svejgaard and Ryder[10] have postulated that HL-A antigens may be cell surface hormone receptors. If so, certain HL-A phenotypes (i.e., HL-A13 and HL-A17), as well as others as yet unidentified) might lead to reduced binding of endogenous catecholamine to the beta receptor. In fact, such a situation might account for the observed propanolol induced, psoriasis-like rashes[11] and the recent observation by Wiley and Weinstein[12] of a sixfold increase in the number of epidermal cells synthesizing DNA induced by intradermal injection of propanolol into uninvolved psoriatic skin. These recent observations by Wiley and Weinstein[12] are potentially important because, as will be discussed below, we believe the reduction in the function of the epidermal cyclic AMP system is central to the misregulated homeostasis of psoriatic epidermis.[8] It will be important to determine whether the elevation in the number of DNA synthesizing epidermal cells in response to propanolol occurs in all psoriatic patients or specifically in those who are HL-A13 or HL-A17 or some as yet unknown HL-A phenotype.

Cyclic Nucleotides in Psoriasis - In 1971 Voorhees and Duell proposed the first working model of a potentially deranged cyclic AMP system in psoriasis.[13] The model was based on the fact that three characteristic features of the lesional epidermis of psoriasis are glycogen accumulation,[8] decreased terminal differentiation[8] and increased proliferation.[14] In other tissues and experimental systems, cyclic AMP was capable of reversing

these three abnormalities.[8] In the model, reduced cyclic AMP function could either initiate a lesion, permit maintenance of a lesion, or both.[13] Since the absence of these three abnormalities describes a normal epidermis, a key postulate of the model is that normal function of the cyclic AMP system is necessary for normal epidermal physiology, a major feature of which is regulated growth. In 1973 this model was modified to include the observed elevation of cellular cyclic GMP levels in association with induced cell proliferation.[15] Our present formulation suggests that reduced cyclic AMP function and elevated cyclic GMP function, or altered function associated with a decrease in the cyclic AMP/cyclic GMP ratio, is central to the deranged epidermal homeostasis characteristic of a psoriatic lesion. Since 1971 these possibilities have been explored in several laboratories, including our own, have been recently reviewed in detail elsewhere,[16] and will only be summarized here.

At this time no direct, clear data are available which demonstrate a role for either cyclic AMP or cyclic GMP in epidermal physiology. However, a considerable amount of indirect, inferential and circumstantial evidence points to a probable highly significant involvement of cyclic AMP in epidermal physiology. This, along with the fact that cyclic AMP is a pivotal effector molecule in the physiological regulation of most tissues, makes it probable that epidermis will not turn out to be an exception. The role of cyclic GMP in cell physiology in general is currently unclear and thus the role of cyclic GMP as a proliferative effector in epidermis is highly speculative at this time. An elevation of cyclic AMP in epidermis *in vitro* thus far has proven to be an inhibitory signal for cell proliferation.[8,17,18] However, due to the high concentrations of cyclic AMP elevating drugs employed in these studies *in vitro*, it is by no means certain what a given cellular level of cyclic AMP does in *in vivo* epidermis. It is possible that the metabolic status of the epidermis can at one time perceive a given cyclic AMP concentration as a proliferative signal and at another time interpret the same cyclic AMP content as an inhibitory proliferative signal. Furthermore, although the cyclic AMP content of a tissue is easy to measure and convenient to think about, it is the net function of the cyclic AMP system in the tissue that is important physiologically. The levels of cyclic AMP and cyclic GMP in psoriasis, although known, are disputed.[16,19] The net function of dysfunction of the cyclic AMP and cyclic GMP systems in psoriasis has never been investigated. Said differently, cyclic AMP dependent protein kinase activity and substrate phosphorylation can be measured in psoriasis. However, the problems in interpreting such data in terms of physiology and/or pathophysiology are enormous. Thus extensive research will be required before any meaningful conclusions can be drawn about the cyclic nucleotide system in either psoriasis or epidermal physiology in general.

Our measurements indicate that the cyclic AMP/cyclic GMP ratio in psoriatic lesions is reduced.[16] The level of cyclic GMP is consistently increased[16] whereas depending on the study, the level of cyclic AMP is either slightly increased,[19] normal[19] or modestly reduced.[16] Perhaps the most important observation is that whereas most metabolic parameters in psoriasis are unequivocally elevated[20,21] as is the case with any acti-

vated metabolic state, the lesional content of cyclic AMP is either the same or 25% above or below that of uninvolved tissue. Our view is that the cyclic AMP system in a psoriatic lesion needs to be stimulated by additional cyclic AMP to generate an antiproliferative signal in the lesion. Said differently, the normal lesional cyclic AMP content or even a 25% elevation may be insufficient to stimulate the lesional cyclic AMP system to a level of activity which is capable of restoring normalcy to the lesion. This view in pharmacological terms means that if we use drugs or hormones to generate more cyclic AMP in the psoriatic lesion, a therapeutic result may be achieved.

This approach is strengthened by several studies. As previously mentioned, work from a number of laboratories[8,17,18,22-24] utilizing adult rodent or human epidermis in culture, indicates that cyclic AMP elevation is capable of inhibiting cell division. One study demonstrated the inhibitory effects of cyclic AMP elevating agents on psoriatic epidermal cells *in vitro*.[23] Two reports claim the *in vitro* stimulation of epidermal differentiation by cyclic AMP elevation.[18,25] These reports[18,25] although consistent with our cyclic nucleotide model of psoriasis are inadequate because they do not adequately document the direct and specific role of cyclic AMP as an inducer of epidermal differentiation. Cyclic AMP has also been shown to stimulate the disappearance of glycogen from psoriatic lesions *in vitro*.[8]

Cyclic AMP is certainly but one possible regulator of proliferation and differentiation in epidermis. However, in several systems cyclic AMP appears to play a major role in growth and differentiation as exhaustively reviewed by Friedman.[26] Also, cyclic AMP regulates glycogenolysis in several tissues.[8] We thus feel justified in postulating therapeutic effects by raising psoriatic lesional cyclic AMP, which based on the aforementioned studies should reduce proliferation, induce differentiation and reduce lesional glycogen content and in so doing should normalize the lesion. To this end, several preliminary studies have already appeared. Stawiski *et al*[27,28] in two separate double blind clinical experiments have shown the superiority of papaverine and d,l-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724) when used topically in comparison to control vehicle treatment. RO20-1724 at a concentration of 1% was significantly better than 1% papaverine cream but neither was as good as topical triamcinolone. However, experimental determination of the correct drug concentration, dosage schedule and pharmaceutical formulation could transform either of these tools for research into clinical drug candidates.

In less well controlled studies topical theophylline, with or without topical dibutyryl cyclic AMP or a cyclic AMP analog, has shown modest efficacy in psoriasis.[29,30] An uncontrolled clinical trial of oral theophylline is said to have demonstrated beneficial results in psoriasis.[31] In two uncontrolled clinical studies of intramuscular dibutyryl cyclic AMP, studies inspired by our earlier biochemical studies on cyclic nucleotides in psoriasis,[13] Chinese physicians in Shanghai considered the drug effective in 80% of 69 patients treated.[32,33] In the Chinese studies, reported side effects were insignificant and thus it should be possible to duplicate their study but using a double blind design. In view of the excellent re-

ported results with minimal side effects, such a controlled trial in our view should have a high priority.

In light of these promising reports, the strong circumstantial evidence that cyclic nucleotides are significantly involved in the pathophysiology of psoriasis, and the certainty that it will be several years before definitive data are available on the role of cyclic nucleotides in psoriatic pathophysiology, it seems reasonable to suggest that further clinical pharmacology utilizing the cyclic AMP approach is warranted at this time. This seems especially important since conventional systemic treatment (anticancer drugs, glucocorticoids and oral psoralen plus ultraviolet light type A) either has known serious side effects or serious potential sequelae. Since cyclic AMP is the second messenger of many peptide hormones and neurotransmitters, one's initial reaction to cyclic AMP therapy for any disorder might be negative on the grounds that cyclic AMP would activate multiple organs. However, local and systemic therapy of asthma, which in our view is probably largely the result of elevated cyclic AMP levels, has been used for years with great benefit and minimal side effects when administered by a physician versed in the pharmacology of sympathomimetic agents.

Clearly several approaches to the cyclic AMP therapy of psoriasis are possible. Epidermis accumulates cyclic AMP *in vitro* when exposed to isoproterenol, epinephrine, norepinephrine, E-type prostaglandins, adenosine, dopamine, salbutamol[34] and histamine (H_2 type).[35] Recent studies by Duell, Terpenning and Collins[36] show that epidermis probably contains a beta receptor of the $beta_2$ type. Thus clinical pharmacology using $beta_2$ catecholamine agonists, such as salbutamol or terbutaline, with or without a cyclic AMP phosphodiesterase inhibitor either topically or systemically appears experimentally feasible. When contemplating such a study, it must be remembered that agonists which stimulate the accumulation of cyclic AMP in several tissues lead to both agonist specific and nonspecific tachyphylaxis.[37] According to one group of investigators, the psoriatic epidermal beta receptor is relatively insensitive to cyclic AMP accumulation induced by beta catecholamine (epinephrine) under *in vitro* conditions.[38] If this abnormality can be confirmed by other groups, it may suggest that the beta catecholamine approach is less desirable than another agonist such as a suberythema or minimal erythema concentration of topical prostaglandin of the E series. Other potential candidates for evaluation are the newly discovered prostacyclin,[39] which is an extremely potent stimulator of cellular cyclic AMP accumulation,[40] or a more stable analog of prostacyclin.

Cyclic AMP phosphodiesterase inhibitors, with or without an appropriate agonist are also candidates for clinical experimentation. In preliminary work, which requires confirmation, we have reported greater low Km cyclic AMP phosphodiesterase activity in psoriatic lesions than in normal appearing areas in six patients.[41] If this is the case in an *in vivo* lesion and is not an artifact of tissue preparation for biochemical analysis, a cyclic AMP phosphodiesterase inhibitor may be an excellent choice. We have shown that epidermal cyclic AMP phosphodiesterase is inhibited by caffeine, theophylline, diazepam, papaverine and RO20-1724.[34] The methyl xanthines are poor inhibitors, whereas papaverine and RO20-1724 are potent

inhibitors leading to pronounced accumulation of epidermal cyclic AMP in vitro. RO20-1724 in certain tissues selectively inhibits the hydrolysis of cyclic AMP vs. cyclic GMP.[42] If we assume that cyclic GMP plays an important role in the psoriatic process, and if the cyclic AMP phosphodiesterase inhibitory selectivity exists in epidermis, RO20-1724 could be an unusually useful agent. In fact, as mentioned above, in preliminary studies we found that topical RO20-1724 produced improvement in 75% to 77% of 42 patients in two separate double blind studies.[28] Presumably, the improvement is the result of elevated lesional cyclic AMP, although other unknown actions of this agent could have produced the beneficial effect.

Tachyphylaxis to agents which raise cyclic AMP was mentioned above.[37] This is a serious concern when considering the potentialities of cyclic AMP therapy for psoriasis. The molecular basis of this tachyphylaxis is poorly understood, and what is understood is beyond the scope of this review.[37,43] Tachyphylaxis in this review should be considered to be synonymous with the term "tissue desensitization". This phenomenon has been known at the clinical level for years in asthma therapy. By proper choice of drug dose and time of administration, it should be possible to determine the proper therapeutic regimen in psoriasis as it has been determined in asthma. By attention to these details, it may be possible to experimentally determine an efficacious drug regimen for psoriasis. However, lack of attention to the tachyphylaxis issue by employing protocols without flexibility of drug dose and time of administration, may cause an experimental agent to be discarded as not sufficiently potent. It is unlikely that a regimen of cyclic AMP therapy for psoriasis will be discovered by the so-called "quick and dirty" approach.

Another critical point is the interrelation between cyclic AMP and glucocorticoids. It is quite clear from a number of studies[44-47] that in order for cyclic AMP to regulate cell function in a normal manner, a certain critical amount of glucocorticoid must also be exerting or have exerted its action in the same cell. In general, it appears that cyclic AMP and glucocorticoid induced metabolic signals move the cell in parallel directions but their modes of action appear to be different.[46] In fact, in a series of elegant somatic cell genetic analyses, Coffino et al[47] have shown that no mutable step is common to cyclic AMP and glucocorticoid in the case of lymphoma cell cytolysis produced by these two agents. The necessity of glucocorticoid for normal cyclic AMP function has been termed the "permissive effect" of glucocorticoids.[44] It is not known whether this permissive effect can be extrapolated to therapeutics. However, it is possible that for additional cyclic AMP to function in a cell, there must also be additional glucocorticoid. Therefore, it might be that elevation of cyclic AMP in the psoriatic lesion would have, at best, a modest effect in the absence of added glucocorticoid. This concept can best be examined by applying to the skin a suboptimal concentration of a cyclic AMP elevating agent such as theophylline or RO20-1724, plus an amount of glucocorticoid which, by itself, has no appreciable effect in psoriasis. One could then observe whether this combination would have a greater therapeutic effect than either agent alone. If so, such a combination would have the advantage of efficacy without the side effects of long term gluco-

corticoid therapy (thinning and tearing of the skin) which are frequently seen when glucocorticoids alone are used at therapeutic doses.

Arachidonic Acid Transformations - Only infrequently do the patches of psoriasis coalesce to produce total body involvement. The coalescence occurs by the extension of centrifugally enlarging patches. Therefore, it is unlikely that cyclic nucleotides in the skin are regulated by circulating hormones. If this were the case, it would seem that total body psoriasis would be the rule rather than the exception. We have therefore searched for other factors which would be produced locally and also regulate the metabolism of cyclic nucleotides. Psoriasis is induced in the genetically predisposed person[1] by cutaneous trauma.[3] Trauma to skin and other tissues causes the release of free arachidonic acid from membrane bound phospholipids. Arachidonic acid is then transformed to a series of biologically potent substances, several of which have been discovered by Samuelsson and which are the subject of an excellent review.[48] Prostaglandins E_2 and $F_{2\alpha}$ (derivatives of arachidonic acid) are elevated only 40% and 86%, respectively, in lesional tissue.[49,50] However, another derivative of arachidonic acid, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) and free arachidonic acid itself are elevated 82- and 26-fold, respectively.[49] Arachidonic acid can stimulate the formation of cyclic GMP[51,52] and may be able to inhibit the formation of cyclic AMP. Prostaglandins of the E series cannot only stimulate the synthesis of cyclic AMP,[49] but on prolonged bathing of a tissue (such as a psoriatic lesion) in prostaglandin, may do just the opposite (i.e., by tissue desensitization[37] render the tissue refractory to further production of cyclic AMP by prostaglandin). How or if the 82-fold excess of HETE participates in lesional pathophysiology is unknown. Nevertheless, it is of great interest to note that treatment of psoriasis with topical glucocorticoid reduced the lesional content of arachidonic acid and HETE to normal.[50] This presumably is due to inhibition of phospholipase A_2 by glucocorticoid. The net result would be a normalization of all components of the arachidonic acid cascade distal to phospholipase A_2 and any misregulated cyclic nucleotide metabolism produced by cascade constituents.[50] Clearly, new non-glucocorticoid drugs which reduce tissue levels of arachidonic acid and those which would preferentially inhibit the synthesis of HETE by the lipoxygenase will be tried. Perhaps certain of these agents will possess activity against the inflammatory proliferative skin disease psoriasis and other so-called steroid responsive diseases.

Polyamines and Psoriasis - The polyamines (putrescine, spermidine and spermine) have been found to be elevated in all proliferating cells thus far examined.[53] Some unknown but critical level of polyamine is thought to be necessary for a cell to synthesize DNA. Therefore, reducing the polyamine level in a proliferating cell below some critical point can block cell proliferation.[54] Thus we examined the lesions of psoriasis for increased polyamine levels. The levels of all three polyamines[55] and the corresponding three biosynthetic enzymes[56] were elevated in involved areas in comparison with uninvolved areas. Interestingly, treatment of these patients with topical glycocorticoids markedly reduce the activities of the three poly-

amine forming enzymes.[56] Also, alpha methylornithine inhibited the first (ornithine decarboxylase) of these three enzymes, and methylglyoxyal *bis*-guanylhydrazone inhibited the two subsequent enzymes (the putrescine-stimulated and the spermidine-stimulated S-adenosyl methionine decarboxylase). It may be that either these nonsteroidal agents or others can be used to reduce polyamines to a sufficiently low level in psoriatic patches to prevent DNA synthesis or reduce its rate to normal. It is clear that several experimental approaches to the therapy of psoriasis are available, approaches based on modulation of known misregulated molecular mechanisms. We are currently evaluating some of the approaches discussed in this review. and hope to evaluate others as new agents become available.

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Chapter 18. Activators of Dopamine and β-Adrenergic Adenylate Cyclases

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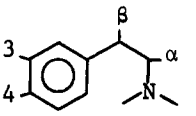
The three major natural catecholamines are epinephrine, from the adrenal medulla, norepinephrine (NE) from specific nerve endings and dopamine (DA) from other nerve endings lacking the enzyme dopamine beta-hydroxylase. These agents are free to act on specific receptors which have been described in pharmacological terms as α,β[1] and most recently DA[2] types. It should be noted that while DA has been found to be a weak agonist of the α and β receptors, NE is a modest agonist of the DA receptor. Many tissues contain β receptors and these are prominantly associated with the relaxation of the smooth muscle of the vasculature and bronchioles as well as the contraction of cardiac muscle[3]. Recent work has shown them to be involved in the proliferation and differentiation of cells[4,5] as well as the release of hormones[6] and enzymes[7]. Dopamine receptors in the periphery are also involved in relaxation of certain vascular beds and the release of hormones but most attention has focused on their role in central nervous system function. Insights into the role of DA in Parkinson's disease and psychosis have emerged and grown through the use of dopamimetic agents like L-DOPA and DA antagonists such as the antipsychotics[2].

Subsequent to the findings of the Sutherland group[8], evidence from laboratories too numerous to list support the idea that the β-adrenergic receptor is coupled to the generation of cyclic AMP (cAMP) while the α receptor, with few exceptions, is not. The picture with the DA receptor is less clear. Recent neurophysiological[9,10] and pharmacological[11] evidence suggests that, as with NE responses, there may be at least two types of DA responses. In analogy with the NE responses, it would not be surprising to find one DA response associated with the generation of cAMP and another not.

DA receptors coupled to adenylate cyclase (AC) have been described for membrane preparations from a number of tissues and these are discussed in a recent review[12]. The availability of a DA sensitive AC made it possible to extend structure-activity relationship (SAR) studies to a level comparable to that carried out with the β system. The availability of both types of AC coupled receptors made it possible to ascribe the activity of a particular agonist or antagonist to interactions at a level considered to be the earliest in a series of biochemical events leading to the physiological responses generally measured in intact tissues or cells. More recently, the binding of agonists and antagonists to membrane fragments has been used as a more direct measure of interactions with the receptor but it should be kept in mind that binding of substances to membranes include interactions other than that of ligand to cyclase receptor[13]. It becomes necessary, therefore, to correlate the data with some response of these membrane fragments and in this regard the AC serves as an excellent model[13]. It should also be kept in mind that binding data do not yet distinguish between agonist and antagonist although some modifications

are being developed which may make that possible[14].

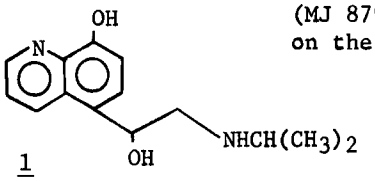
During the last two years a number of papers have appeared which use AC for drug screening or for mechanism of action studies. Both approaches have generated data which can be helpful for the development of SARs as to potency and specificity. This report will discuss agonists and their inactive analogues and derivatives which have been tested on the β and DA adenylate cyclases and attempt to define structural requirements for enzyme activation. Antagonists, other than those which are derivatives of agonists, will not be covered.



Phenethylamines:

I. β-Adrenergic cyclase

A. Aromatic group: Substitution of the 3-OH group of isoproterenol (ISO) with a methylsulphonamido (MSA) group, $-NH-SO_2-CH_3$, yielded soterenol, with equal or greater affinity[15,16,17] but reduced intrinsic activity[16,17]. Inversion of the β-OH lowered potency and its removal lowered it even more[16]. Deoxysoterenol showed only antagonist activity[17]. Other 3-MSA derivatives with a cyclopropane (MJ 8798-1) or a $2,2-(CH_3)_2$-phenethyl (MJ 9184-1) on the nitrogen possessed good agonist activity[17]. The 3-MSA derivative of DA, however, was inactive with the rat red blood cell (RBC) enzyme[15]. The 4-MSA derivatives with or without the 3-OH possessed only antagonist activity[15,16,17]. Another agonist with a nitrogen at position 3 is quinterenol (1)[16].



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Substitution of an hydroxymethyl group for the 3-OH of ISO yielded salbutamol which has agonist activity with frog RBC[16] but not rat ventricle cyclase[18]. The 4-deoxy-ISO (S-40045-9)[16] and 4-deoxy-N-ethyl-NE (S-40032-7)[17] were inactive on the frog RBC enzyme. With the rat RBC enzyme 4-deoxy-α-methyl NE (metaraminol) but not 4-deoxy DA (metatyramine) had agonist activity[19]. Metaraminol and phenylephrine, however, were only antagonists with the frog RBC[17] and rat ventricle cyclase[18]. Interestingly, moving the 4-OH of ISO to position 5 led to reduced but detectable agonist activity[19]. DA was inactive with the RBC enzyme of the frog[16,17] but weakly active with that of the rat[15]. The lack of response to DA by the frog RBC enzyme may be a matter of sensitivity since the affinity of ISO and other agonists appears to be about ten times greater in the rat RBC cyclase[15,16,17].

The methylation of either or both ring hydroxyls of DA reduced agonist activity but did not result in the formation of an antagonist[19]. While a methyl group at position 2 had no effect, a phenyl group markedly reduced activity[19]. Activity was also lost if a methyl group was added to position 5 or 6 of $N,N-(CH_3)_2$-DA or an OH or NH_2 group was added to position 6 of DA[19].

B. Nitrogen substitutions: Alkyl substitution generally increased activity such that isopropyl>ethyl>methyl>H in the β-OH series[15,18]. In the β-deoxy series, the order of potency was methyl>ethyl> isopropyl>propyl>H[19]. The S-isomers of the β-OH series were much less potent with methyl and isopropyl derivatives about equiactive[11,12] and the isobutyl inactive[18]. N,N-dimethyl DA had reduced activity and the addition of a third methyl group led to inactivity[15]. The activities of the β-OH and β-deoxy compounds generally increased with phenethyl substitutions[17,20].

C. Side chain substitution: As indicated above, inversion of the β-OH led to compounds comparable in activity to the β-deoxy analogues but appreciably below that of the R-conformers. It is clear that the β-OH is not essential for β agonist activity. The insertion of an $-OCH_2-$ between the β-carbon and the aromatic ring of ISO (MJ-9910) increased the affinity for the frog RBC[16] cyclase. The observed reduced intrinsic activity is consistant with the β-adrenergic blocking activity usually associated with such side chains[17].

Substitution on the α-carbon has not been extensively studied but addition of a methyl to either DA or NE resulted in enhanced activity[17,19]. In the DA series, the increased activity was confined to the S-conformer[19]. Addition of an ethyl group to the α-carbon of NE had the same enhancing effect as a methyl group but a reduced effect when added to ISO[18].

II. The DA cyclase

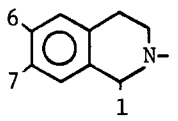
In general, substitutions on the aromatic ring of DA have changed the agonist potencies in a fashion similar to that observed for the β-agonists. None of the alterations increased the activity while a marked decrease was noted with a phenyl group at position 2, a methyl group at positions 5 or 6, an OH, NH_2, or NO_2 at position 6, removal or methylation of either the 3 or 4-OH[13], and a CH_2OH in place of the 3-OH[21]. The 4-deoxy compound, m-tyramine, was reported to have either some[19] or no[22] agonist activity, while metaraminol had none[19]. Substitution of a MSA for the 3-OH group of DA (MJ 7582) reduced activity only slightly[19].

N-Methylation had little effect on the activity of DA[19,22]. The addition of two methyl groups reduced activity to different degrees[19,22]. N,N,N-Trimethyl DA was reported to be inactive[19] or as active as N,N-dimethyl DA[22]. Agonist potency decreased markedly with an increase in size of the alkyl group[19]. Surprisingly, N,N-diethyl DA was slightly more potent than N-ethyl DA and N-isopropyl-N-methyl DA was weakly active compared to the inactive N-isopropyl DA[19]. Interestingly, replacement of the methyl or n-propyl group of N-methyl-N-n-propyl DA with an n-butyl increased the potency close to that of N-methyl DA[23].

The β-OH reduced activity such that the rank order of potency was DA>R-NE>S-NE>>R-ISO[19,22]. Thus the S-conformer of NE does not have the potency of the β-deoxy compound (DA) as noted with the β-system.

Reducing or increasing the length of the side chain by one carbon resulted in a loss of agonist activity[22]. Methylation of the α-carbon also markedly lowered activity[19,22], with the S-isomer being less potent than the R conformer[19].

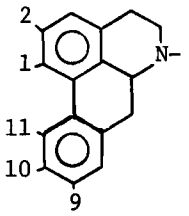
Tetrahydroisoquinolines: It has been postulated that the aldehydic products of the oxidation of ethanol and dopamine can condense with dopamine to form 6,7-dihydroxy-tetrahydroisoquinoline (THI) with a methyl[24,25] or 3,4-dihydroxybenzyl[20] substituent in position 1, respectively.



β-Adrenergic cyclase: Tetrahydropapaveroline (THP), which represents the condensation of DA with 3,4-dihydroxy-phenacetaldehyde, was about as active as NE[20]. The S-isomer was preferred and N-methylation destroyed activity[20]. Agonist activity was also lost if the 6,7-hydroxy groups were methylated or a third hydroxyl was introduced in position 5 of the 3,4-dihydroxybenzyl portion[20]. Activity was not appreciably altered if the benzyl hydroxyls were methylated, replaced by a p-Cl group or only the 3-OH group was removed[20]. Activity was reduced markedly by substituting a methyl, dimethoxyphenyl, or p-Cl-phenethyl group at position 1[20]. The most potent compound of the series and more active than ISO was trimetaquinol which has a 3,4,5-trimethoxybenzyl substituent at position 1[16,20]. The S-isomer was about 1000 x more potent than the R-conformer[20]. In many cases, the potency of a THI derivative was greater than that of its open ring analogue[20]. All β-agonists had reduced intrinsic activity which could be ascribed to their ability to act as antagonists[20], as noted earlier for trimetaquinol[16]. THI compounds without the 6,7-hydroxyls but with dihydroxy or dimethoxybenzyl substituents at position 1 exhibited only antagonist activity[20]. As noted for agonist activity, the S-isomer was preferred and N-methylation reduced activity appreciably[20].

DA cyclase: Most THI derivatives were not DA agonists but several were antagonists. Very weak agonist activity was noted for the N-CH_3 derivative of 1-H and 1-CH_3-6,7-dihydroxy-THI[27] and the nor-1-H compound[22]. Again the S-isomer was more potent but N-methylation tended to increase antagonist activity[20]. A benzyl substituent appears to be important for antagonist activity since the 1-methyl and 1-dimethoxyphenyl compounds were inactive[20].

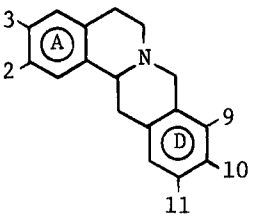
Aporphines: These compounds are of interest because of the well known action of apomorphine on DA systems in vivo[12]. Very little work has been reported with respect to the action of these compounds on β-systems other than the inhibitory action of apomorphine (APO) on the rat RBC cyclase[28]. The action of APO on DA cyclase is one of stimulation at low concentrations and inhibition at high concentrations[15,29] with the result that the intrinsic activity is less than that of DA although



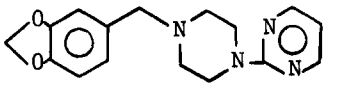
potency is generally enhanced. The rank order of agonist potency was N-allyl APO>2-hydroxy-APO=APO>N-desmethyl APO[15]. The N-n-propyl derivative is another potent agonist[22]. However, removal of the 11-OH or its transfer to position 9 (isoapomorphine) resulted in loss of agonist activity[30]. Methylation of the 10-OH of APO (apocodeine)[20] or of its N-n-propyl analogue abolished agonist activity[30]. The S-isomer of APO[20], the APO analogue with the OH groups at 1,2 rather than 10,11[30] and bulbocapnine, the 1,2-methylenedioxy-10-methoxy-S-conformer of APO, were antagonists[20,30].

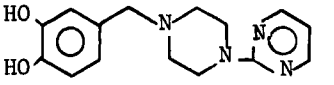
One derivative, (S)-1,2,9,10-tetrahydroxy-noraporphine is a β-agonist[31] whose formation from THP was suggested[32].

Tetrahydroprotoberberines: The formation of tetrahydroprotoberberines (THPB) from THP was also suggested[33]. One such derivative was the 2,3,9,10-tetrahydroxy-THPB which had no β-agonist activity[31]. Racemic 2,3,10,11-tetrahydroxy THPB exhibited β-agonist activity weaker than THP[34]. A number of other THPB derivtives elicited only DA antagonist activity. Among the 2,3-dihydroxy analogues with hydroxyl groups also at positions 9,10 or 10,11, the S-conformer was significantly more potent than the R-isomer[20]. Steric preference was not evident if methoxy groups were in those positions[20]. The presence of a 2,3-methylenedioxy group in addition to methoxy groups at 8,10, as in canedine, did not alter potency significantly[21].



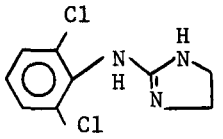
Miscellaneous compounds:

ET-495 

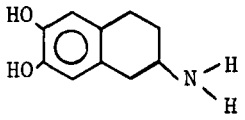
S-584 

ET-495 (piribedil) demonstrated dopaminergic activity *in vivo*[35] but failed to activate the DA-AC[36,37] except for a slight stimulation after a 10 min. preincubation with rat caudate slices[37]. Based on the rationale that removal of the methylene bridge was essential for activation of the DA receptors, S-584 was prepared and found to be almost as active as DA on AC of rat striatum[36] and caudate nucleus of the cat, rabbit, cebus and rhesus monkeys[37]. S-584 was inactive on the DA and β-AC of the cebus monkey frontal cortex and much weaker than DA on the anterior limbic cortex AC[38]. Some uncertainty exists concerning the identification of S-584 as a *pure* DA agonist.

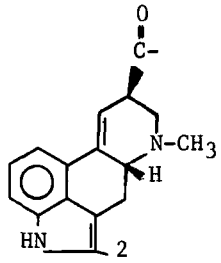
Clonidine: This compound bears no resemblance to a catecholamine but is believed to possess α-agonist activity[39]. It weakly activates the AC of the frontal cortex but not the anterior limbic cortex of the cebus monkey[38].



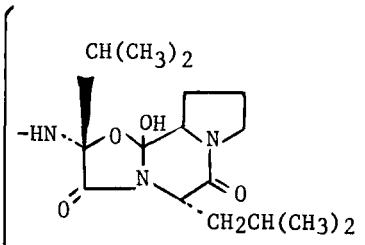
2-Aminotetraline: 6,7-Dihydroxy-2-aminotetraline (ADTN) has been tested on the DA cyclase and found to possess strong agonist activity[36] despite its atypical dopaminergic responses in vivo[40].



Ergot alkaloids: Though many of the ergot alkaloids are dopaminergic



D-Lysergic Acid



: 2-Br-α-ergocryptine (CB-154)

$-N(C_2H_5)_2$: LSD

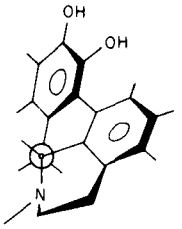
$-NH-\underset{\displaystyle CH_3}{\underset{|}{CH}}-CH_2OH$: Ergometrine

in vivo[41], particularly in inhibiting the secretion of prolactin[42], only a few have been tested on the DA-AC system. LSD has been found to both activate and inhibit rat striatal AC[43,44] while 2-Br-α-ergocryptine (CB-154)[45] and ergometrine[46] only inhibited the system. Much more work with other ergot alkaloids is needed to clarify their relationship with the DA-AC.

Polymerically immobilized catecholamines: NE and ISO joined to agarose beads through the 6 position of the aromatic ring instead of the side chain nitrogen possessed β-agonist activity which could be accounted for by the presence of free catecholamines cleaved from the agarose by the myocardium membrane preparations[47]. Those complexes not cleaved by frog RBCs were β-antagonists with ghosts but not intact cells[47].

Conclusions: One of the questions which may be answered with the information presented above concerns the conformations of the natural agonists, DA and NE, at the DA and β-receptors. The receptor microenvironment will most certainly influence the nature of the interacting forces and thus the conformations. According to the concept of activation of AC, the agonist induces a conformational change in the receptor, thereby activating the catalytic portion of the system[48]. It is also reasonable to expect that the receptor can induce a conformational change in the agonist. The natural catecholamines have a great deal of structural flexibility making it extremely difficult to know the activating conformations at the receptor. Some of the agents discussed here have much less flexibility because critical groups are fixed in ring systems. Such compounds can provide better insights into the conformations necessary for activating AC.

Two classes of compounds with limited flexibility but almost perfect specificity are the aporphines (DA-agonists) and tetrahydroisoquinolines (β-agonists). By examining their structures through Newman projections (2 and 4) certain structural differences emerge which may define the conformational preference of the phenethylamines (3)[20] at the receptor. In APO (2) the nitrogen is trans to the catechol function and the m-hydroxyl projects toward it. In contrast, the nitrogen, of a THI (4) is gauche to the catechol function and the m-hydroxyl points away from it.



(R,-) APOMORPHINE

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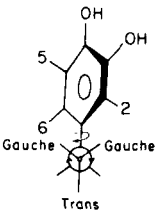
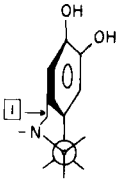


3,4 - DIHYDROXYPHENETHYLAMINES

3



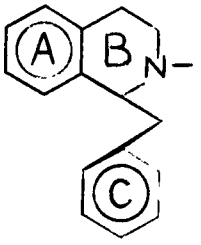
6,7-DIHYDROXYTETRAHYDRO-ISOQUINOLINES

4

The 6,7-dihydroxy THI structure may represent the preferred conformation at the β-adrenergic receptor but derivatives lacking the benzyl moiety at position 1 are inactive despite good activity of their open chain analogues. A reasonable explanation of this is that the binding of the aromatic ring of the catecholamine agonist positions the molecule for interactions involving ring hydroxyls and side chain nitrogen. The flexibility of the side chain permits the nitrogen to find its binding site on the receptor and initiate the activating conformational change. When the movement of the receptor reaches a state accomodating a THI-like conformation, the catalytic unit is signaled to cyclize ATP. If a THI is the agonist, the nitrogen is unable to reach its binding site, because

as part of a heterocyclic ring its movement is restricted. A benzyl group at position 1 would possess sufficient flexibility to locate and interact with the receptor at an hydrophobic site and direct its movement toward an activating conformation. The nitrogen can now interact with its binding site and complete the activation[20]. Since the active THI compounds lack an hydroxyl group at the carbon β to the nitrogen, a β-hydroxy is not essential but may serve to stabilize the binding or promote the establishment of a gauche conformation[20].

The ability of 1-benzylated THI (5) to inhibit both DA and β-receptors has been attributed to the existance of two phenethylamine structures that can provide both gauche (6) and trans (7) conformations[20].



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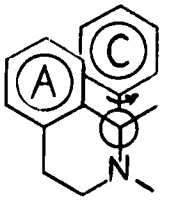

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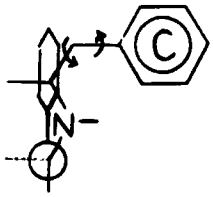

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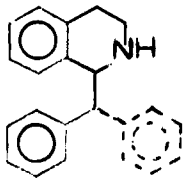
Since both the aporphine and THPB derivatives of THP can activate the β-receptor suggests that the benzyl ring is acceptable in either of the two positions outlined in 8. This may be rationalized in terms of the groups within the receptor that would likely interact with the aromatic ring under consideration. A phenylalanyl residue (9) would be free to occupy the same positions as the benzyl ring and thus would be able to interact with the appropriate aromatic ring of the active aporphine and protoberberine[34].



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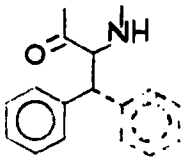

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The substances discussed above have helped to develop models of the structural requirements for activation of the DA and β-receptors of adenylate cyclase. The model is by no means complete and there is some difficulty in trying to make the structures of S-584 and ADTN fit. If their catechol functions were superimposed on that of APO the remaining nitrogens would be separated by as much as several angstrom units. Since it is not clear that the position of the nitrogen in APO is preferred, the displacement from that position may not be as serious as first thought. In any event, synthetic efforts aimed at answering some of the questions raised here will ultimately help to provide a better understanding of the interactions which occur between these agonists and their receptors.

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Chapter 19. Modulation of the Arachidonic Acid Cascade

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Introduction – Since the discovery over 12 years ago that the then recently discovered prostaglandins are biosynthesized from arachidonic acid[1-3], numerous studies have helped elucidate the details of this transformation. Recent studies have shown that arachidonic acid serves as a precursor for a virtual cascade (Scheme 1) of acidic lipids, each possessing distinct and profound pharmacological activities. While PGE_2 and $PGF_{2\alpha}$ have been established as local modulators of a variety of physiological processes, the physiological role of the endoperoxides PGG_2 and PGH_2, which have been found to possess potent pharmacological activities of their own, including the aggregation of platelets[4-9], contraction of smooth muscle[8,10] and a possible role in acute inflammation[11,12], is less clearly established.

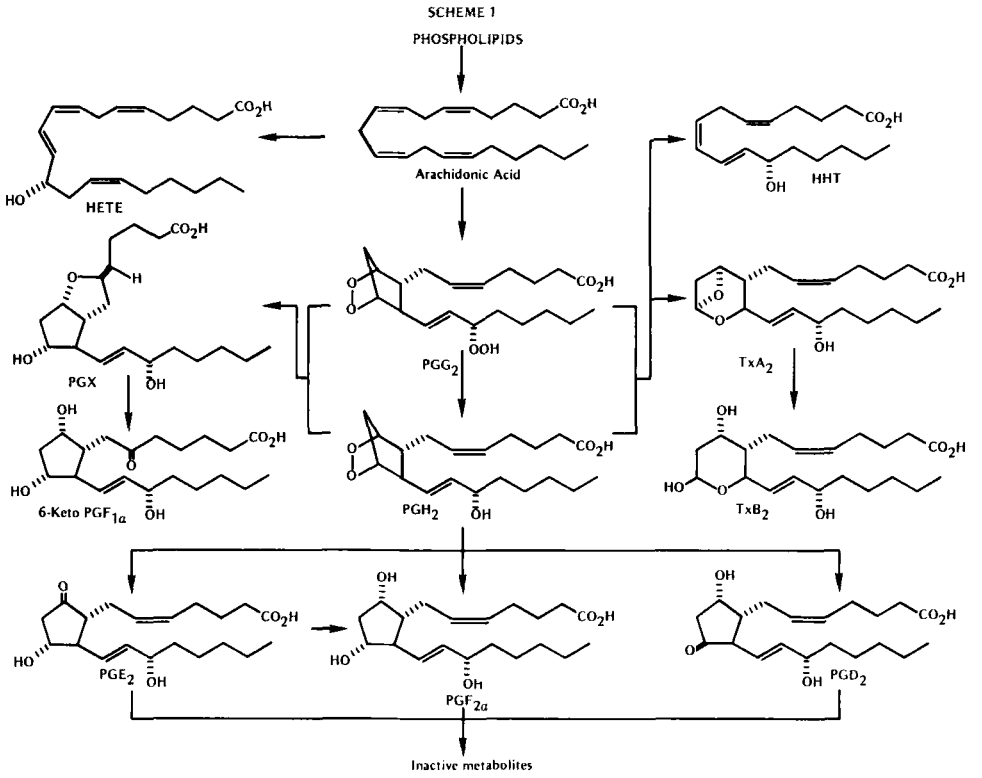
Samuelsson and co-workers discovered that platelet microsomes convert the endoperoxides into the labile thromboxane A_2[14] (TxA_2) which rearranges into the stable thromboxane B_2 (TxB_2). TxA_2, which aggregates platelets and contracts arterial smooth muscle[19-21], has been postulated to play a pivotal physiological role in platelet aggregation[14] and in maintaining arterial tone[133]. Consistent with the finding[15] that PGH_2 and TxA_2 are the constituents of the previously isolated[16] rabbit aorta contracting substance (RCS), thromboxanes have been isolated from challenged, sensitized guinea pig lungs[17] and from phagocytosing polymorphonuclear leucocytes[18]. TxB_2, initially reported to be inactive, has been shown to stimulate the release of slow reacting substance of anaphylaxis (SRS-A) from challenged, chopped guinea pig lung[17], and to possess chemotactic activity[22]. TxB_2 has also been isolated from guinea pig brain homogenates[23] and carrageenin-induced granuloma[134].

Recently, Vane and his colleagues reported that arterial or venous tissue converts the endoperoxides into the novel, labile PGX (prostacycline)[24-26]. As PGX is both a potent inhibitor of platelet aggregation[27] and an arterial vasodilator[135], a homeostatic control of both platelet aggregation and arterial blood flow by PGX and TxA_2 has been proposed[28,135]. PGX is converted into the stable 6-keto $PGF_{1\alpha}$ which has been detected in rat stomach[29,30] and brain homogenates[31], sensitized guinea pig lungs[32], carrageenin induced granuloma in rats[34] and, probably, rabbit heart[33].

The endoperoxides decompose into the fatty acid HHT[35]. This transformation is probably a non-enzymatic artifact and no biological activity has been reported for this compound. Another fatty acid, HETE, formed from arachidonic acid by a platelet lipoxygenase[36], has been found to exhibit chemotactic activity for neutrophils[37] and eosinophils[38] *in vitro*. The recent suggestion that arachidonic acid is a precursor for SRS-A, while provocative, needs further experimental verification[13].

This plethora of structures and attendant activities provides the medicinal chemist with a wealth of possibilities for the design of therapeutic agents. One approach, agonists designed to exploit the potential therapeutic uses of prostaglandins, was the subject of last year's review. An alternative approach to achieving selective *in vivo* prostaglandin activities is to modulate the enzymes which control tissue levels. The following is a review of selected agents which have been shown to modulate the numerous enzymatic steps in the arachidonic acid cascade.

Phospholipids → arachidonic acid – Arachidonic acid metabolites appear not to be 'stored' within cells. Their biosynthesis depends upon the appearance of substrate at or near the microsomal synthetase complex(es). According to current thinking[39], arachidonic acid is stored in the phospholipid fraction of the cell from which the free fatty acid is liberated by the action of a phospholipase A_2.



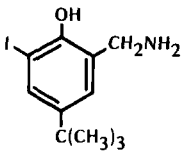
In contrast to the lack of effect on prostaglandin biosynthesis in broken cell preparations[40], anti-inflammatory steroids exhibit potent inhibitory effects on prostaglandin production in rheumatoid synovial[41,42], $HSDM_1C_1$ mouse fibrosarcoma[43] and MC5-5 mouse fibroblast cultures[44], as well as in rat synovia and rabbit uvea *in vivo*[45]. Corticosteroids also block the release of prostaglandins in guinea pig lungs perfused with RCS-releasing factor[46]. Available data are consistent with a site of action prior to the release of arachidonic acid[136].

Bradykinin releases prostaglandins from phospholipids[49] in a variety of systems[47], an effect thought to augment the activities of this peptide. That bradykinin activates phospholipase A_2, either directly or indirectly[136], is suggested by the inhibition of bradykinin-induced prostaglandin release by the phospholipase inhibitor mepacrine[48]. The local anaesthetic tetracaine[50] and psychotropic drug meprobamate[51] have been reported to inhibit prostaglandin biosynthesis at the phospholipase level.

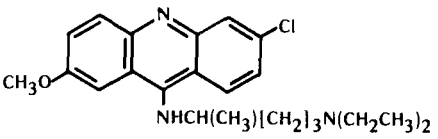
Arachidonic acid → endoperoxides – The hypothesis[52-54] that non-steroidal anti-inflammatory (NSAI) agents owe their pharmacological activity to inhibition of prostaglandin biosynthesis (PGBS) has been the subject of several reviews[55-57]. Inhibition of purified cyclo-oxygenase (endoperoxide synthetase)[58] from bovine seminal vesicle (SV) microsomes by aspirin and indomethacin supports the hypothesis that this enzyme is the site of action of NSAI agents. The precise mechanism of inhibition, however, may differ for various classes of NSAI compounds[59,60].

Several workers[61-63] have recently questioned the hypothesis that primary prostaglandins are obligatory mediators of acute inflammation. Support for this view is found in the report that MK-447, which possesses *in vivo* anti-inflammatory activity, stimulates prostaglandin synthesis *in vitro*[12]. MK-447, as well as aspirin and phenylbutazone, significantly decrease the amount of PGG_2 produced *in vitro*, which suggests that PGG_2, rather than PGE_2, is a pro-inflammatory factor[12].

The observation that both aspirin and dexamethasone depress the acute inflammatory response to carrageenin in rats depleted of arachidonic acid[64] suggests that activities other than PGBS inhibition contribute to the anti-inflammatory effects of both steroids[114] and NSAI agents[115]. Inhibition of PGBS by a series of NSAI agents *in vitro* appears to be better correlated with *in vivo* analgesic effects than with anti-inflammatory activity[137].



MK-447

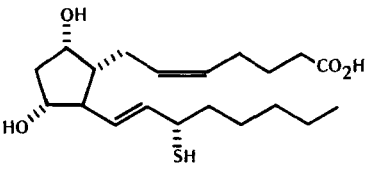


Mepacrin

Other agents reported to inhibit PGBS *in vitro* are the fatty acid eicosa-5,8,11,14-tetraynoic acid[65] and several constituents of cannabis[66-68]. As several phenolic antioxidants inhibit PGBS[69], the activity of the cannabis derivatives may be ascribed to the phenolic moiety. Local anaesthetics[50], tranquilizers[51], tricyclic antidepressants[70], and neuroleptics[70,71] inhibit PGBS, but whether this activity is related to the pharmacological action of these agents must await further study.

Morphine and apomorphine at doses lower than those attainable in the brain *in vivo* stimulate PGBS in bovine SV[72] and rabbit brain[73] homogenates. As this effect is inhibited by chlorpromazine[74], which inhibits apomorphine-induced emesis in the dog, stimulation of PBGS may be responsible for some of the side-effects accompanying the administration of opiates.

The prostaglandin analog 15-mercapto $PGF_{2\alpha}$ (1), its C_{15}-epimer, and both 2-descarboxy-2-mercaptomethyl PGE_2 (2) and $PGF_{2\alpha}$ are potent inhibitors of the cyclo-oxygenase *in vitro*[75]. C_9 and C_{11} mercapto analogs are less potent. Structurally less complex thiols, such as 2,3-dimercaptopropanol, while less potent, also inhibit the purified enzyme.



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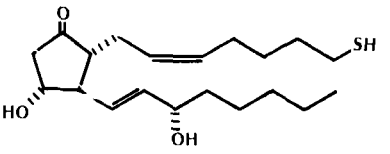
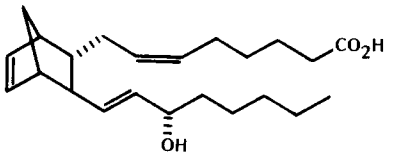


2

Inhibition of PGBS via the cyclo-oxygenase continues to be a valuable tool for defining potential mechanisms of actions of therapeutic agents[76]. Conversely, known PGBS inhibitors are being evaluated in pathological conditions thought to be characterized by excessive prostaglandin production, such as Bartter's syndrome (aspirin)[77], dysmenorrhea (naproxen)[78], bleeding due to IUD's (indomethacin)[116] and diarrhea accompanying thyroid medullary carcinoma (nutmeg and indomethacin)[79].

Endoperoxides → primary prostaglandins – Unlike inhibition or stimulation of the cyclo-oxygenase, modulation of the enzymes responsible for the conversion of PGH_2 into PGE_2, $PGF_{2\alpha}$ or PGD_2[138] would be expected to selectively effect production of only one prostaglandin. However, only the unstable PGE isomerase has been separated from the PGBS complex[81]. The finding that endoperoxide analog **3** selectively decreases synthesis of PGE_2 in ram SV[82], while sodium aurothiomalate and auroglucose inhibit $PGF_{2\alpha}$ synthesis in bovine SV[83], suggests that these compounds inhibit the PGE isomerase and PGF reductase, respectively.

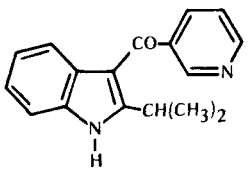


3

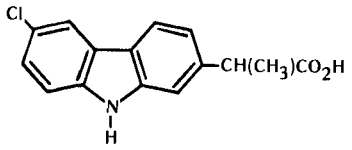
Release of primary prostaglandins from perfused or chopped organs stimulated by a variety of agents has been investigated, but these studies suffer from an inability to determine if the effects observed are directly on the PGBS system or are caused by some other mediator. Such studies also provide no direct information concerning which specific step in the biosynthetic pathway is being effected. Still, these studies provide valuable information concerning the possible interrelationship of biologically active compounds with prostaglandins. Histamine releases $PGF_{2\alpha}$ predominantly from guinea pig lung parenchyma (via H_1-receptors) and PGE_2 from trachea (via H_2-receptors)[84]. Bradykinin releases PGE_2 from bovine arteries and $PGF_{2\alpha}$ from veins[85]. Since, as mentioned above, bradykinin stimulates phospholipase A_2, this finding suggests that arteries and veins selectively biosynthesize PGE_2 and $PGF_{2\alpha}$. Angiotensin I, II and III release prostaglandins (probably PGE) from the perfused rabbit mesenteric vascular bed[86]. Since the pressor response to angiotensin II in the rat preparation is inhibited by indomethacin and restored with PGE_2[88], prostaglandins may modulate the vasoconstrictor effects of angiotensin II.

L-Ascorbic acid selectively inhibits $PGF_{2\alpha}$ synthesis from arachidonic acid in lung homogenates[89]. An α-tocopherol deficient diet stimulates PGBS non-selectively in coagulating rat blood[90]. Biogenic amines stimulate $PGF_{2\alpha}$ synthesis in rat cerebral cortex slices[91], an effect attributed to non-enzymatic reduction of the endoperoxides. A similar non-enzymatic effect has been ascribed to copper dithiols[92], suggesting that care must be taken in interpreting PGBS data as to enzymatic or non-enzymatic processes. Overall, little progress has been made in identifying selective modulators of the primary prostaglandins.

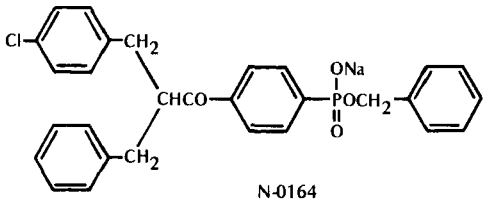
Endoperoxide → thromboxane A_2 – Inhibitors of phospholipase A_2 or cyclo-oxygenase also inhibit the formation of TxA_2. However, comparative studies using a SV cyclo-oxygenase and a platelet thromboxane synthetase have found phenylbutazone and naproxen to strongly inhibit PGBS without any effect on TxA_2 formation[93]. Indomethacin is 20 times more effective in inhibiting PGBS[93], benzydamine 2.5 times more effective in inhibiting TxA_2 synthesis[93] and L-8027 much more potent in inhibiting TxA_2 synthetase than PGBS[139]. Other PGBS inhibitors like mefenamic acid, phenelzine, sulfinpyrazine and RO 20-5702 are inhibitors of TxA_2 formation in human platelets[94], but relative potencies are not known. N-0164, in addition to being a prostaglandin antagonist, inhibits the conversion of PGG_2 to TxA_2 by human platelet microsomes at doses not affecting the cyclo-oxygenase[95].



L-8027



RO 20-5702



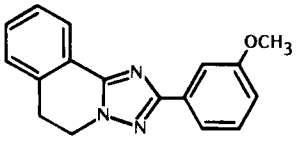
N-0164

Endoperoxide → PGX – Unlike the cyclo-oxygenase and thromboxane synthetase, PGX synthetase is not inhibited by agents such as indomethacin, benzydamine and phenelzine[27]. The enzyme from swine aorta microsomes is, however, inhibited by tranylcypropamine, spontaneously oxidized arachidonic acid, and most strongly by 15-hydroperoxy arachidonic acid (15-HPAA). This has led to the suggestion that TxA_2 (from platelets) and PGX (from vascular epithelium) are pivotal in maintaining physiological platelet and/or vascular homeostasis, imbalance of which (e.g. vascular injury or high concentrations of lipid peroxides) causes platelet aggregation and arterial constriction leading to thrombosis[96] (see Chapter 9).

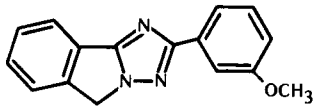
Modulators of metabolism – As the primary prostaglandins are rapidly inactivated metabolically[97], modulation of the enzymes responsible should either increase or decrease endogenous prostaglandin levels. The initial step in the catabolism of most prostaglandins[98] is oxidation of the C_{15}-hydroxy moiety by the enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH)[99]. Large variations in activity of PGDH have been observed in kidney[100] and lung[101] of rats of different ages. PGDH from rabbit lung but not kidney or spleen increased strikingly during pregnancy[102]. Increased PGDH activity was also found in deciduomal and myometrial tissue from pseudopregnant rats[103]. Reduced PGDH activity has been found in spontaneously hypertensive Wistar Okamoto-Aoki rats just prior to an increase in blood pressure[104]. These data allow the conclusion that some pathological conditions suggestive of aberrant prostaglandin levels may be due to defective PGDH and not defective PGBS.

Aberrant levels of PGDH activity can be modified. Thus, hydrocortisone reverses excessive renal PGDH in adrenalectomized rats, while 17β-oestradiol reverses increased PGDH activity in ovariectomized rats. Since both steroids are without effect *in vitro*, the mechanism remains uncertain[105]. Purified swine kidney PGDH has been inhibited by relatively high doses of thyroid hormones[106].

Among NSAI agents[99], indomethacin inhibits the rat kidney PGDH[107]. Imipramine and desipramine, which activate, and furosemide, ethacrymic acid and xylocaine, which inhibit isolated swine kidney PGDH, all appear to act at a site distinct from the substrate or co-enzyme binding sites[108]. Carbenoxolone inhibits PGDH from human gastric mucosa and also inhibits the biosynthesis of $PGF_{2\alpha}$ in guinea pig kidney[109]. L-10503 and L-10492 administered subcutaneously to rats and hamsters at specific times of gestation are potent antifertility agents[110]. These compounds appear to exert their effects directly on the utero-placental complex, perhaps by selectively inhibiting the metabolism of $PGF_{2\alpha}$. A series of prostaglandin analogs have been found to inhibit human placental PGDH *in vitro* [147].



L-10503



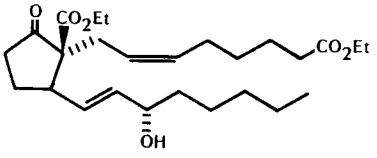
L-10492

The enzyme 9-ketoreductase, which has been identified in kidney[111], vascular tissue[140], brain[112] and skin[141], converts PGE_2 to $PGF_{2\alpha}$. Physiological control of this enzyme is suggested by changes in its activity in rabbit renal cortex in response to changing salt load[142]. Certain biogenic amines (serotonin, norepinephrine, dopamine, epinephrine)[112] stimulate this enzyme, while furosemide and ethacrynic acid[113], as well as indomethacin[107], are inhibitors.

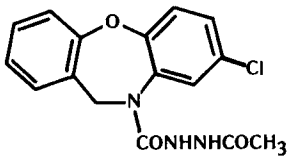
Prostaglandin biotransport – Transport of prostaglandins across several biological barriers (e.g. blood-brain, vagina, kidney and lung) appears to be an energy dependent, active process capable of functioning against a concentration gradient[117]. This finding has prompted the evaluation of compounds as potential transport inhibitors. Thus, probenecid, iodipamide and bromcresol green decrease the clearance of $PGF_{2\alpha}$ from rabbit brain[118], decrease renal clearance of $PGF_{2\alpha}$ in rabbits[119] and reduce lung uptake of prostaglandins in rats[117,120]. The observation that di-4-phloretin phosphate (DPP) inhibits the uptake of prostaglandins into lung tissue[120] suggests that inhibition of pulmonary metabolism of PGE_2 and $PGF_{2\alpha}$ by DPP in rabbits[121] may be due to inhibition of transport.

Endproduct antagonists – As PGE_2, $PGF_{2\alpha}$ and, presumably, thromboxanes, endoperoxides and PGX bind to distinct receptors, the design of selective receptor antagonists has been undertaken. The bulk of studies to date has examined three types of antagonists: dibenzoxazepines (e.g. SC-19,220), 7-oxaprostanoids and polymers of phloretin, the results of which have been reviewed[122-124]. In general, the efficacy, selectivity and competitive nature of these antagonists vary markedly with the *in vitro* system used and little *in vivo* activity has been observed.

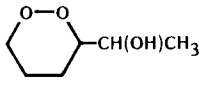
The phenyl phosphonate N-0164 antagonizes PGE_2 and $PGF_{2\alpha}$ on several smooth muscle preparations and prevents PGE_2 induced diarrhea in mice[131]. The prostanoid HR-546 antagonizes PGE_2 induced smooth muscle contractions with an ED_{50} 100 times lower than that of 7-oxa-13-prostynoic acid[125].



HR-546



SC-19,220



4

The observation that morphine inhibits c-AMP formation stimulated by PGE_1 led to the hypothesis[126] that inhibition of a prostaglandin-sensitive adenyl cyclase forms the biochemical basis of the analgesic action of opiates. Morphine also prevents PGE_1-mediated inhibition of the aggregation of human platelets[127] and the positive chronotropic effects of PGE_1 and PGE_2 on guinea pig atria[128]. Similar to morphine, leucine- and methionine enkephalin inhibit PGE_1 stimulated c-AMP formation in hybrid neuroblastoma cells[143].

Δ^9-THC inhibits[132] abdominal contractions induced by PGE_1 in mice. Meclofenamic acid[144] and methyl xanthine phosphodiesterase inhibitors[145] were reported to be prostaglandin antagonists *in vitro*. The observation that chloroquine and related drugs antagonize the action of PGE_2 on the rat mesenteric vascular bed[129] prompted the successful clinical use of chloroquine in closing the ductus arteriosus in neonates[146]. The report[130] that the cyclic peroxide 4 inhibits platelet aggregation induced by PGH_2 ether analogs suggests that this compound may be an endoperoxide antagonist.

Conclusions – Extensive studies have identified a variety of arachidonic acid metabolites with profound biological effects. Identification of the enzymes responsible for the biosynthesis and metabolism of these compounds provides an opportunity for selective modulation of the endogenous levels of these lipids. Examination of the effects of a number of pharmacological agents has given insights into their possible mechanism of action. The demonstration that certain drugs affect endogenous prostaglandin levels has led to the evaluation of these agents in disease states hitherto not considered. Much opportunity, however, remains, for the design and development of truly selective inhibitors or stimulators of the enzymes of the arachidonic acid cascade. Accelerated efforts in this direction can be expected in the future.

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Chapter 20. Recent Advances in the Etiology and Treatment of Disorders of Lipid Metabolism

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Introduction - This review will present recent developments on the etiology and treatment of atherosclerosis, hyperlipidemia and cholelithiasis. The other major lipid disorder, obesity, has recently been reviewed as to pathogenesis and treatment,[1] new efficacy trials and mechanism of action studies on anorectic agents,[2-5] and appetite regulation in normal and abnormal states.[3,6]

Etiology of Type II Hyperlipoproteinemia - In a study of the inheritance of xanthomatosis and hyper-β-lipoproteinemia, the segregation pattern satisfied the criteria for autosomal dominant inheritance, but not for a polygenic trait. The genetic analysis demonstrated that hyperlipoproteinemia Type IIa and Type IIb were the same disease.[7,8] Defective removal of low density lipoproteins (LDL) from the circulation produced the elevated LDL and plasma cholesterol observed in Type II hyperlipoproteinemia. This defect appeared to be related to the decreased number of LDL receptors on cell surfaces (for reviews see 9, 10), although a change in the permeability of the cell membrane to cholesterol may also play a prominent role.

Leukocytes from subjects with familial hypercholesterolemia responded to incubation in a lipid-depleted medium with a higher activation of sterol synthesis and an enhanced induction of HMG-CoA reductase compared to leukocytes from controls. This increase in sterol synthesis and HMG-CoA reductase was correlated with a loss of cholesterol into the medium. A phospholipid-protein-cell interaction was necessary to induce the reductase. Thus, elevated serum cholesterol levels in familial hypercholesterolemia may be attributed to increased cholesterol loss from cells,[11,12] due to a lack of feedback regulation in this condition.

Atherosclerosis and Plasma Lipids - Lipoprotein lipases play a critical role in the metabolism of lipoproteins and thus may be involved in atherogenesis. Hypercholesterolemia in the cholesterol-fed rabbit was attributed to the accumulation of chylomicron remnants, which may be formed on the aorta wall by lipoprotein lipase and deposited in the deep layers of the arterial wall without prior release into the blood stream.[13] On this basis, cholesterol-rich lipoproteins in plasma may be the product rather than the cause of the atherogenic process. However, the defect in Type III hyperlipoproteinemia (broad-β disease) may be ineffective removal of chylomicron remnant particles from the arterial wall,[14] due to a failure of the liver to recognize such particles.[15]

The recent completion of the Coronary Drug Project, a secondary prevention trial in men with a proven previous myocardial infarction, failed to show an effect for nicotinic acid or clofibrate on either plasma cholesterol levels or mortality.[16] A primary prevention trial of cholestyramine resin in Type II hyperlipoproteinemic men is in progress.[17] Hypolipidemic agents which are more effective than nicotinic acid and clofibrate are

needed. However, such drugs may require extensive testing to show efficacy in reversing atherogenesis.[18] Arteriography provides unequivocal proof of lesion regression. In recent reports, lesion regression was confirmed with this method following control of hyperlipidemia with clofibrate and/or other drugs.[19,20] However, only in rare cases is this direct method justified. Objective evidence to validate regression may come in the future from the use of bioengineering techniques.[21]

Experimental atherosclerosis can be reversed by dietary means. A high-cholesterol, high-fat diet combined with arterial injury produced atherosclerosis in swine[22,23] and monkeys.[24] The lesions regressed when the animals were removed from the atherogenic diet and placed on a normal diet.[22,23] Chronic hyperlipidemia itself may produce the primary endothelial injury that initiates this process of atherosclerosis.[24]

The Relationship of Damage to the Arterial Endothelial Lining, Platelet Aggregation and Arteriosclerosis - Injury to the endothelial lining of the artery caused an immediate platelet response; platelets adhered to the subendothelial connective tissue at the sites of injury, aggregated, lost their granules and exhibited decreased survival time.[25,26] Recent studies from the Karolinska Institute and Wellcome Research Laboratories have provided a biochemical rationale for the role of platelets in the genesis of arteriosclerosis (see chapters 9 and 19). Aspirin which inhibits platelet aggregation is currently undergoing a large scale study for its effect on coronary heart disease.[27] However, since the substance responsible for platelet aggregation is apparently thromboxane A_2, a specific thromboxane A_2 inhibitor might have greater therapeutic utility than aspirin.[28] Prostacyclin, which is formed in arterial endothelial cells, not only dissociates platelet aggregates, but also causes relaxation of smooth muscles.[29] This relaxation is a desirable effect since it may decrease phagocytosis by endothelial cells and thereby prevent further narrowing of the arterial lumen.[30]

Hypolipidemic Agents - Several reviews have documented recent advances in hypolipidemic drugs.[11,31] Clofibrate significantly decreased serum triglycerides in Type IIb patients and cholesterol in Type IIa and IIb subjects.[32] Unexpected elevations in LDL were observed after clofibrate administration to Type IV subjects.[33] The drug induced myopathy[34,35] in patients with renal failure and intrahepatic cholestasis.[36] In recent mechanism of action studies, clofibrate 1) augmented post-heparin plasma lipoprotein lipase activity,[37,38] 2) diminished serum clearing factor lipase activating activity,[38] 3) interfered with very low density lipoprotein (VLDL) formation, packaging and release in the liver,[39] 4) inhibited lipolysis[40,41] and enhanced free fatty acid reesterification in adipose tissue,[41] and 5) modified serum proteins and components of the human hemostatic system.[42] Additionally, insulin may be an important mediator of the hypotriglyceridemic effect of clofibrate.[43-45]

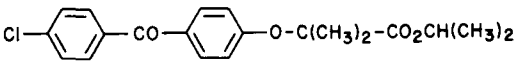
Clinical studies confirmed halofenate to be an effective hypotriglyceridemic and hypouricemic agent.[46,47] Recent mechanism of action studies (earlier investigations were reviewed recently[48]) demonstrated

that halofenate inhibits hepatic triglyceride synthesis,[49] and reduces the ratio of insulin to glucagon in the circulation.[50] Halofenate inhibited platelet aggregation to a greater extent than did clofibrate.[51] The clinical efficacy of gemcadiol was shown in Type II, III, IV and V patients.[52] Its hypotriglyceridemic activity in rats was ten times greater than that of clofibrate.[53] 780SE produced marked hypotriglyceridemic activity without any hypocholesterolemic activity or hepatomegaly;[54] inhibition of triglyceride synthesis was also demonstrated.[55] Tibric acid suppressed triglyceride levels selectively but induced hepatomegaly.[56]

The efficacy of probucol in lowering serum cholesterol levels was confirmed in Type II patients.[57] Interestingly, no effect on cholesterol synthesis, absorption or excretion has been observed.[58] This drug has recently gained FDA approval. Neomycin was as effective as cholestyramine in decreasing cholesterol levels in Type II subjects, but was better tolerated.[59] In rats neomycin produced hypocholesterolemia without altering cholesterol turnover.[60] Clinical trials on the efficacy of bile acid sequestrants as hypocholesterolemic agents have been conducted with cholestyramine,[61] colestipol[62-64] and Secholex.[65,66] Clinical studies suggest that β-sitosterol may be useful as a hypocholesterolemic agent.[67-70] Xanthinol nicotinate produced significant hypolipidemic effects in Type V subjects without causing flushing; chylomicrons and VLDL decreased while LDL increased significantly.[71] Other nicotinic acid derivatives demonstrated good hypolipidemic activity but induced flushing.[72-74]

Intravenous administration of 7-ketocholesterol decreased the uptake of cholesterol into rabbit aorta.[75] 5α-Cholest-8(14)-en-3β-ol-15-one suppressed serum cholesterol levels and hepatic cholesterol synthesis.[76] S-8527 significantly reduced serum cholesterol by inhibiting the hepatic synthesis of lipoprotein fractions carrying cholesterol.[77] Metformin produced only a slight reduction of plasma cholesterol levels in rabbits fed a high cholesterol diet. However, it markedly decreased aortic cholesterol esters and the atheromatous process, with a simultaneous change in the composition of VLDL.[78,79]

New Hypolipidemic Agents - LF 178 (Lipanthyl®, 1), a member of a series of alkylcarbonyl- and benzoyl- phenoxycarboxylic acids,[80] was more potent than clofibrate in hyperlipidemic and normal animals.[81] Clinical efficacy was demonstrated in Type IIa, IIb and IV hyperlipoproteinemia;[82] the compound was more potent than clofibrate after one month treatment. A multicenter trial confirmed LF 178 as a well-tolerated hypocholesterolemic and hypotriglyceridemic agent.[83] Tiadenol lowered cholesterol and triglycerides in long-term trials in Type IIa and Type IIb patients.[84]



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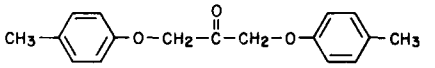

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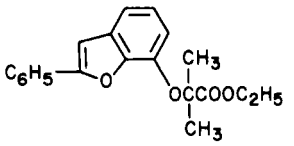
Several 1,3-bis(substituted phenoxy)-2-propanones possess hypocholesterolemic activity in rats; the 1,3-bis(p-methylphenoxy)-2-propanone

(2) lowered serum triglycerides and glycerol.[85] 2-Hexadecanone significantly reduced serum cholesterol levels in rats;[86] whereas 2-octanone demonstrated hypocholesterolemic and hypotriglyceridemic activity.[87] Rats receiving 2,8-dibenzyl-cyclooctanone demonstrated hypocholesterolemia which was associated with estrogenic activity.[88] Structure:activity relationships of a number of benzofuran derivatives demonstrated either hypotriglyceridemic, hypocholesterolemic or both activities. Derivatives 3 and 4 were as active as clofibrate in reducing both triglycerides and cholesterol,[89] whereas 5 selectively decreased cholesterol, and 6 and 7 effectively suppressed



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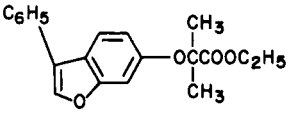

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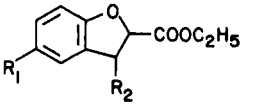
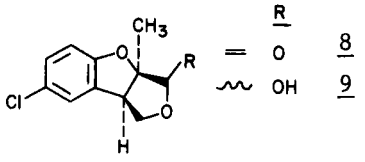
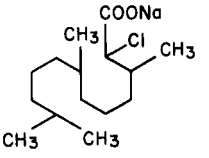


R_1	R_2	
C_6H_5	H_2	5
Cl	=O	6
C_6H_5	=O	7

only triglyceride levels in rats.[90] A tricyclic clofibrate-related lactone (8) and its precursor (9) reduced triglycerides and/or cholesterol depending on whether normal or Triton hyperlipidemic rats were employed.[91] Compounds 10 and 11 significantly decreased cholesterol, triglycerides and free fatty acids in both normal and hyperlipidemic rats.[92] An isoindo-



R		
=	O	8
≈	OH	9



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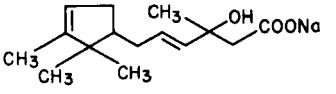
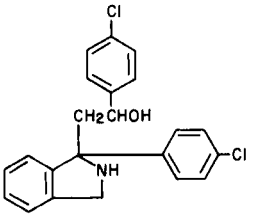


11

line derivative (12) and certain analogs significantly lowered serum cholesterol, triglycerides and phospholipids in the rat, and serum cholesterol in the dog.[93] Hypo-β-lipoproteinemia [decrease in heparin precipitating lipoproteins (LDL and VLDL) and increase in high density lipoproteins] was demonstrated with U-41792 (13) in several animal species.[94]



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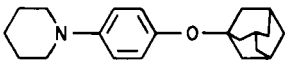
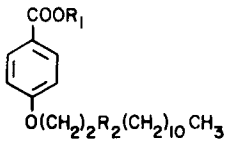
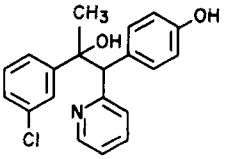


13

A number of substituted benzenesulfonic acid esters and p-chlorophenoxyisobutyric acid esters produced hypolipidemic activity.[95] Among a series of p-alkoxybenzoic acids, enhanced hypotriglyceridemic and hypocholesterolemic activity was observed with oximino- (14) and chloro- (15) substituents.[96] Hypocholesterolemia was observed after the administration of 16 to rats.[97] Tetronic 701, a polymeric surfactant, also lowered serum cholesterol; the tetrabenzoate of Tetronic 701 is of particular interest, since it produced comparatively less growth depression.[98] Two linoleamide derivatives, (-)N-[α-phenyl-β-(p-tolyl)ethyl]linoleamide (PTLA) and N-(α-methylbenzoyl)linoleamide, suppressed serum and liver cholesterol levels in rats and inhibited cholesterol absorption by interfering with the hydrolysis of cholesteryl esters in the intestine.[99-101]

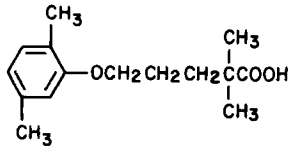


	R_1	R_2	
	CH_3	NOH ‖ C	14
	H	Cl | CH	15



16

The oral administration of 3-hydroxy-3-methylglutaric acid (HMG) prevented the alcohol-induced postprandial elevation of serum lipids and β-lipoproteins in man.[102] CL-719 (17) produced a dose-dependent suppression of plasma triglycerides and cholesterol in Type IV subjects.[103] A valeric acid derivative (18) decreased cholesterol synthesis in rat liver in vivo but exerted no inhibitory effect on HMG-CoA reductase;[104] a glutaric acid derivative (19) was a very potent inhibitor of HMG-CoA reductase from rat liver.[105] Fatty acid synthesis was significantly suppressed in vivo by RMI 14,514, a furoic acid derivative; the benzoic acid and acetic acid benzyl ester analogs (RMI 13,640 and RMI 14,425) were less active.[106]



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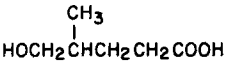

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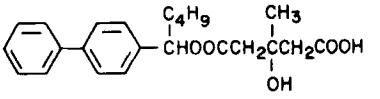


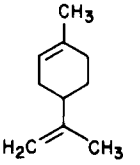
19

There are several new approaches to the treatment of hypercholesterolemia. Sucrose polyester (SPE), a mixture of hexa-, hepta- and octaesters of long-chain fatty acids, is a noncaloric, unabsorbable synthetic fat that decreased cholesterol absorption in rats.[107] Each replacement of 1% dietary triglyceride with SPE resulted in a 1.2% decrease in cholesterol absorption. After treatment with SPE, total plasma cholesterol and LDL cholesterol were significantly reduced in normal subjects but not significantly in hypercholesterolemic subjects.[108] A novel technique based on the principle of affinity chromatography, involving an extracoporeal system for the reduction of plasma cholesterol through the specific removal of LDL by heparin-agarose, was reported.[109]

Anticholelithiasis Agents - Several reviews discuss the current views on the pathogenesis and management of gallstones.[110-112] Obesity has been reported to be accompanied by a three-fold increase in the occurence of gallstones;[1] this increase may be explained on the basis of enhanced hepatic secretion of cholesterol in obese subjects, resulting in the production of lithogenic bile.[113] Similarly, an increased prevalence of cholelithiasis, likely due to the enhanced cholesterol saturation of bile, has been observed with the administration of conjugated equine estrogens[114] and oral contraceptives.[115]

Clinical studies confirmed the efficacy of chenodeoxycholic acid (CDC) in producing complete or partial dissolution of gallstones, with a concomitant reduction of the lithogenicity of bile and increase in the ratio of biliary CDC to cholic acid and deoxycholic acid (for review see 9). High dietary cholesterol levels antagonized the therapeutic efficacy of CDC.[116] Additional studies suggested that continuous CDC therapy appears necessary, since the biliary cholesterol saturation index remained low for only three weeks after withdrawal of CDC.[117] Hepatotoxicity, apparently produced by lithocholic acid (LC), the bacterial metabolite of CDC, continued to be reported in animal species which lack an adequate capacity to sulfate LC.[118] However, no evidence of hepatotoxicity, either enzymatically or morphologically, was observed in patients administered CDC chronically at therapeutic doses, except for an occasional elevation of serum transaminases.[119-122] A controlled, single-blind study failed to confirm any hypotriglyceridemic activity of CDC.[123] Sulfation is known to lead to rapid intestinal excretion, thus preventing LC accumulation in the enterohepatic circulation. Effective sulfation of LC (> 75%), particularly its glycine conjugates, may explain the lack of hepatotoxicity in humans.[124,125]

d-Limonene (20), introduced through a choledochal catheter, was effective in dissolving the gallstones remaining in the common bile duct after surgery.[126] Its choleretic activity and ability to decrease the biliary lithogenic index in the dog were attributed, in part, to its metabolites.[127] Zanchol, a hydrocholeretic agent (21) reduced the cholesterol saturation of bile when administered to patients who had undergone cholecystectomy.[128] Phenobarbital, which was ineffective as an anticholelithiasis agent[129,130] may diminish the efficacy of CDC in dissolving gallstones.[131]



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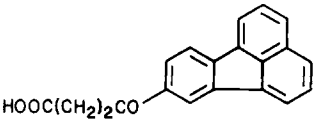

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Chapter 21. Drug Metabolism

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Introduction – This chapter summarizes significant contributions from the drug metabolism literature of the past year, with the objective of illustrating how pharmacokinetic and biotransformation information can assist in the discovery and development of new drugs. The format follows closely that of last year[1]. The influence of structural alterations on drug disposition, metabolic events that lead to pharmacologically active or toxic substances, advances in novel or little studied biotransformations, and species differences in metabolism are discussed.

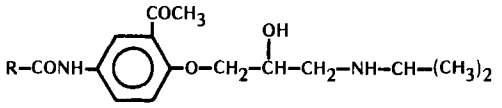
A number of reviews and textbooks within the broad scope of drug metabolism merit attention. The role of drug metabolism in medicinal research has been reviewed[2], as well as information on the biliary excretion of foreign compounds[3], pharmacokinetic aspects of drug interactions[4], and developmental patterns of the hepatic monooxygenases[5]. A text is now available which provides an overview of recent cytochrome P-450 chemistry and biology[6]. Current knowledge on the utility of pharmacokinetics in clinical drug usage has been summarized[7], and earlier volumes on foreign compound metabolism[8] and drug isolation and identification techniques have been updated[9]. New monographs review the disposition of psychotherapeutic agents[10], as well as progress in selected areas of drug metabolism[11]. A guide to the design of drug assays has been assembled[12]. An important new work discusses the current understanding of basic processes involved in drug metabolism[13].

The hydroxylation of amobarbital[14,15] and breath analysis of aminopyrine demethylation[16] are proving to be useful tests to determine a patient's capacity for hepatic drug oxidation. The increased use of hepatocyte preparations demonstrates their utility as models for drug metabolism studies[17-20]. The important role of extrahepatic drug metabolism has been further explored in intestine (analgesics)[21] and lung (nicotine)[22]. A brain capillary permeability technique has been described which allows determination of the relative penetration of drugs into the brain[23]. The well established method of combined gas chromatography-mass spectrometry, more recently supplemented with field desorption mass spectrometry, continues to be extremely useful in solving a variety of drug metabolism problems[24]. The simplicity, sensitivity and specificity of radioimmunoassays has led to the development of a number of practical drug assays in biological fluids[25-31]. Good agreement with well established methods makes this technique increasingly attractive for clinical monitoring of large numbers of samples.

A number of recent reviews and symposia have addressed the relationship of drug plasma concentrations with pharmacological effect, and the utility of pharmacokinetic data in rational drug therapy[32-38]. In general, pharmacological effects correlate more closely with drug plasma levels than with dosage. However, efficacy may not correlate with drug plasma concentrations when an active metabolite is formed or when transport to the site of action is exceedingly slow. For many agents, drug level determinations are helpful in adjusting dosage during therapy. Drug level information is also often desired by the clinician when the expected therapeutic effect is not seen or when toxic manifestations attributable to drug treatment are encountered.

Relationships between Structure and Pharmacokinetics – Inactive l-propoxyphene enhanced the analgesic activity of d-propoxyphene in the rat, apparently by decreasing hepatic extraction of the latter[39]. Rapid stereospecific uptake of the β-adrenergic blocking agent l-propanolol by heart tissue was observed in the rat following dl-propanolol administration[40]. An active acetylated metabolite (**2**) of the β-adrenergic blocking agent acebutol (**1**) achieves plasma levels in patients up to ten times those of the parent[41], possibly due to differences in half-lives. The potent analgesic activity of meperidine (**3**) and N-substituted homologues in rodents has been attributed to more efficient brain penetration of these agents despite weaker affinity to

the opiate receptor than morphine[42]. Compared to cocaine (4), the less potent stimulant pseudococaine (5) has a shorter half life in rats, while the potent metabolite norcocaine (6) has a somewhat longer half-life[43]. The more potent S(-)enantiomer of the anticoagulant phenprocoumon (7) possesses a smaller volume of distribution, is less rapidly cleared from plasma and is more highly bound to serum albumin than the R(+) isomer[44].



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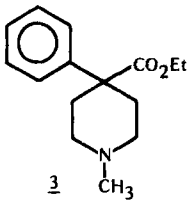
2, R = CH_3



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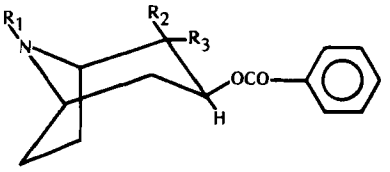
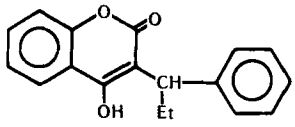


	R_1	R_2	R_3
4,	CH_3	CO_2CH_3	H
5,	CH_3	H	CO_2CH_3
6,	H	CO_2CH_3	H



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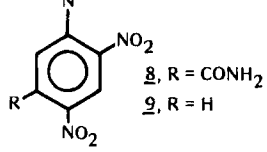
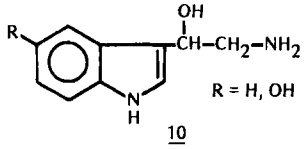
The important role of drug-protein binding in determining drug action has been reviewed[45,46]. Tissue binding of the cardiac glycosides and barbiturates is closely correlated with their lipid solubility[47]. The major parameter involved in serum binding of tricyclic tranquilizers and antidepressants has been shown to be affinity of the charge transfer complex[48]. Serum binding of rutin flavanoids increased in proportion to the number of unsubstituted phenolic groups[49]. S(+)-Benzetimide was stereoselectively bound and more slowly mobilized to and from the binding site of guinea pig atria than the R(-) enantiomer[50], consistent with the more potent atropine-like properties of S(+)-benzetimide.



8, R = $CONH_2$
9, R = H

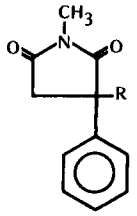


R = H, OH

10

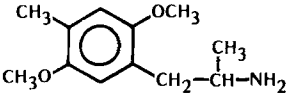
Relationships between Structure and Biotransformation – Within a series of aliphatic amines (C_4-C_{10} and C_{13}), the rate of monoamine oxidase activity was maximum with n-hexylamine[51]. The carboxamide 8 was metabolically more stable in rats than compound 9[52]. β-Hydroxylated tryptamines (10) were much more slowly deaminated to corresponding ethanediols than non-β-hydroxylated tryptamines[53]. The position of methoxy substituents in O-methyl catecholamines influences the route of metabolism, as L-4-O-methyldopa was extensively N-acetylated, while L-3-O-methyldopa was metabolized in man and rat to corresponding pyruvic, lactic and acetic acid derivatives[54]. The superior anticoagulant activity of methsuximide (11) compared to phensuximide (12) was attributed to more rapid metabolism of the latter[55]. A major metabolite of 13 was the corresponding 2-hydroxypropionic acid, whereas the latter was formed from 14 in

only minor amounts[56]. Deuteration of the methyl substituents of caffeine at the 1- or 7-positions suppressed demethylation at the deuterated site in favor of demethylation at the nondeuterated position[57].



11, R = CH_3
12, R = H

13, R = CH_2–CH=CH_2
14, R = CH=CH–CH_3



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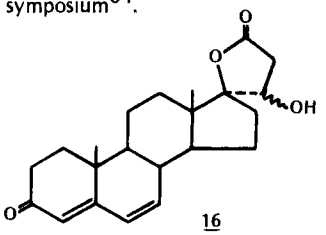
A number of recent studies extend the observation of stereoselectivity of drug metabolism. The inactive d-isomer of propanolol was metabolized in rats with a two-fold shorter plasma half-life than l-propanolol[40]. The anti-inflammatory agent l-α-methylfluorene-2-acetic acid was isomerized in dogs to the d-enantiomer[58], thus being another example of stereospecific metabolic inversion[1]. Whereas the individual R and S enantiomers of **15** were metabolized at similar rates, the half-life of the more active R isomer was prolonged in the racemic mixture, perhaps due to inhibition of metabolism of the R isomer by the S isomer[59]. A similar effect was observed with the R and S enantiomers of amphetamines[60], which further illustrates that racemates may exhibit a biological profile different from that anticipated on the basis of the activity of the component enantiomers.

The metabolism of a variety of drugs has been correlated with their physicochemical properties[61] and the concept of regioselectivity in drug metabolism has been reviewed[62].

Metabolic Activation — Numerous publications have appeared on the subject of biotransformation of drugs to active metabolites or to reactive intermediates which manifest toxicity. The pharmacological activities of the metabolites of 58 drugs and the consequences of their accumulation in patients with renal disease have been reviewed[63]. Mediation of drug toxicity by reactive metabolites was the subject of a recent symposium[64].



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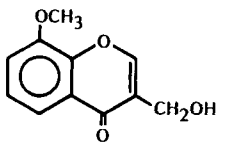

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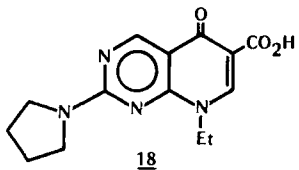

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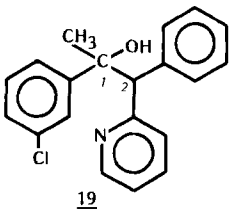
Carboxylic acid metabolites derived from cocaine (**4**) and norcocaine (**6**) persist in dog brain after the parent drugs have been cleared, and may play a role in acquired drug hypersensitivity[65]. Metabolism of potassium canrenoate to 20-hydroxycanrenone (**16**) may contribute to the antimineralocorticoid activity of canrenone[66]. A 3-carboxyl metabolite appears to be responsible for the antianaphylactic activity of **17**[67]. Pyrrolidine ring-hydroxylated metabolites of piromidic acid (**18**) are more potent antibacterial agents than the parent drug[68]. Compound **19** and its major 2(4-hydroxyphenyl) metabolite in the rat[69] exhibit comparable hypocholesteremic effects in this species, suggesting that metabolism may be responsible for the species-dependent activity of **19**. The concept that circulating glucuronides may function as drug reservoirs was advanced from studies with the β-adrenergic blocking agent bunolol[70]. Carbinol (**21**) and ketone (**22**) metabolites of bufuralol (**20**) possess β-adrenergic blocking activity comparable to **20** and

exhibited longer plasma half-lives in man[71]. Evidence was presented that metabolism to mono-N-methylated products is essential for antitumor activity in a series of aryldialkyltriazenes (23)[72]. The antitumor activity of 1-nitro-9-(3'-dimethyl-n-propylamino)acridine may be due to an intermediate N-hydroxylated metabolite[73].



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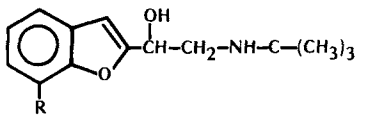
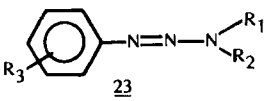


R = CH_2CH_3(20), $CHOHCH_3$(21), $COCH_3$(22)



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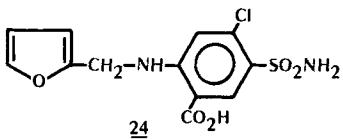


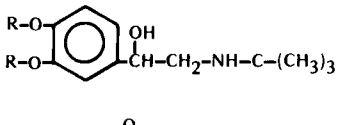
24

The assumption that epoxides derived metabolically from aromatic or olefinic compounds exert adverse biological effects may not always be justified[74-76]. Epoxidation of the 10,11-double bond of centrally active tricyclic agents, now considered a common and important metabolic process with these drugs, affords stable epoxides which lack mutagenic and cytotoxic activity (carbamazepine, cyproheptadine, cyclobenzaprine)[74]. Studies with the carcinogen trans-4-acetylaminostilbene reveal that the α,β-epoxide and dihydrodiol metabolites are non-mutagenic[76]. The presence of a 10,11-epoxide in human brain following carbamazepine administration suggests that this metabolite is responsible for the anticonvulsant activity of this drug[77]. Evidence for apparently stable arene oxide metabolites of centrally active tricyclic drugs has also been obtained[78,79].

Numerous studies have demonstrated an apparent relationship between metabolite formation and toxicity. The N-hydroxylation of phenacetin may play a role in drug-induced hepatic necrosis[80]. Similarly, N-hydroxylation may mediate acetaminophen hepatotoxicity[81]. Acetylhydrazine and isopropylhydrazine, metabolites of isoniazid and iproniazid, may initiate hepatotoxicity through covalent binding of an electrophilic intermediate (see Chapter 32)[82].

A reactive intermediate involving the furan ring of furosemide (**24**) may be responsible for the hepatic necrosis produced by this diuretic agent[83]. Covalent binding of isoproterenol[84] and rifampicin[85] to macromolecules can occur following phenol oxidation to adrenochrome and quinone, respectively; reduction of oxidation products back to parent drug has also been observed. An association of drug toxicity of halothane[86] and antipyrine[87] with irreversible binding of reactive intermediates to liver proteins has been discussed.

A number of new prodrug esters with improved pharmacological properties compared to the parent drug have been described. The di-p-toluate ester of N-t-butylarterenol (bitolterol, **25**) is a longer-acting bronchodilator with reduced cardiovascular side effects[88] than the unesterified drug[89]. In an extension of earlier work, a series of apomorphine diesters (**26**; dipropionyl, diisobutyryl, dipivaloyl, dibenzoyl) elicited prolonged duration of neuropharmacological effects in rats; duration of action increased with the size of the ester group[90].



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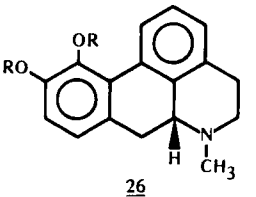

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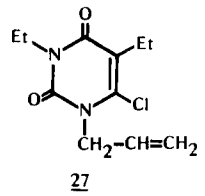
New Pathways of Metabolism – Investigations on novel or little studied biotransformations warrant attention, as the information may help predict the fate of new drugs. Methylmercapto replacement of the chloro substituent in the antiviral agent acluracil (**27**)[91] and sulfoxidation of the pyridyl moiety of bromazepine (**28**)[92] may involve mercapturic and cysteine conjugation; displacement of chlorine by a methylsulfonyl group in the herbicide propachlor (**29**) has recently been reported to derive from cysteine and mercapturic conjugates[93]. A general scheme analogous to that proposed earlier for aliphatic amines has been proposed for the metabolic N-oxidation and subsequent transformations of primary and secondary aromatic amines[94]. Nitro metabolite formation has been reported for MK-251 (**30**)[95] and 1-phenyl-2-propanone oxime[96]. Thus, intermediate oxime metabolites of amphetamine-like compounds may be expected to be further metabolized to nitro compounds. The amine function of **30** is metabolized to a small extent also to the corresponding methyl ether, the first apparent example of this type of metabolic transformation[95]. Direct evidence has been presented for metabolic double bond hydration, an uncommon event usually masked by the reverse process; thus, hydrated metabolites of ethyl apovincaminate (**31**)[97] and d-limonene (at C-1) (**32**) were identified in several species, including man[98].



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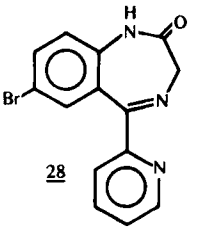

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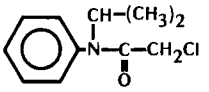

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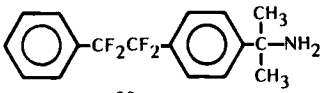

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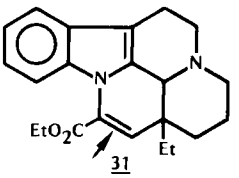

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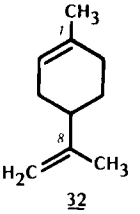

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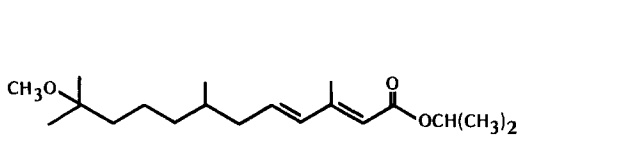
A new pathway of primary amine metabolism (glucuronyl carbamate formation) requiring the insertion of a carboxyl group between amino group and glucuronic acid, has been proposed for the new antiarrhythmic agent alanyl-2,6-xylidine (tocainide)[99]. Novel fatty acid conjugates of the 11-hydroxylated metabolites of Δ^9-THC and Δ^8-THC have been identified that are retained in rat tissues for long periods[100]. The identification of these metabolites suggests that other drugs may be retained in the body in this manner. Metabolic reduction of the dienoate system of the insect growth regulator methoprene (**33**), and incorporation of the carboxylic acid into glycerides and cholesterol esters, has been observed[101]. Tripelennamine is another example of a drug that is extensively metabolized to a quaternary ammonium N-glucuronide[102]. The anti-inflammatory agent **34** is metabolized predominantly to a novel acyl glucoside in the mouse[103].



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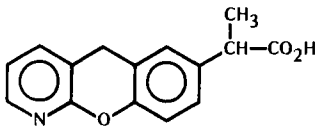

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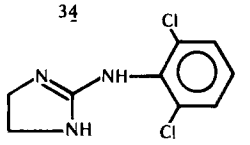

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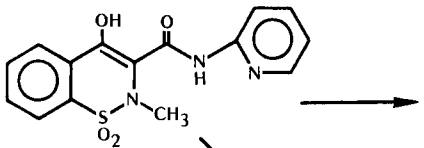
Clonidine (**35**) is metabolized in rats and dogs by ring cleavage to 2,6-dichlorophenylguanidine[104]. N-acetylated amino acid metabolites of mephenesin and dipropylacetate in rats are thought to arise from a mechanism involving oxidation to hydroxylated carboxylic acids which enter general transamination pathways[105]. Unlike the related agents sudoxicam and isoxicam[1], piroxicam (**36**) is appreciably metabolized by pathways involving cyclodehydration (to **37**), and benzothiazole ring contraction to *o*-sulfobenzimide (**38**)[106]. Metabolite **39** is found in patients intoxicated with phenobarbital or glutethimide[107]. Chromone **17** undergoes γ-pyrone ring scission to hydroxyacetophenones[108].



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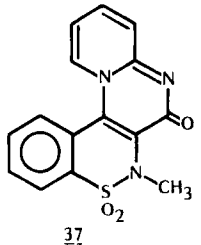

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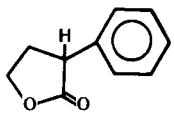

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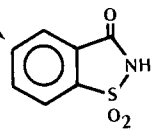

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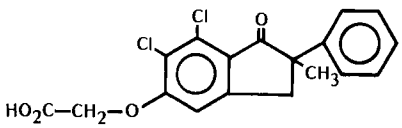
Comparative Metabolism – Pharmacokinetic principles that can assist in an understanding of species variations in drug action have been recently reviewed[109,110]. Comparative metabolism studies of antineoplastic drugs were conducted in several species to assess their suitability as alternatives to the rhesus monkey in safety studies[111]. The domestic pig appears to be a suitable model for oxisuran in man[112]. The rhesus monkey, rather than dog or rat, more closely resembles man in the overall disposition of spironolactone[113]. However, metabolism of glutethimide differs in monkey (rhesus or cebus) and man[114]. Rats and dogs extensively metabolize bethanidine, whereas in man appreciable metabolism does not occur[115]. The new diuretic MK-196 (**40**) is minimally metabolized in rat, dog and rhesus monkey, but, as in man, is extensively metabolized in the chimpanzee[116].



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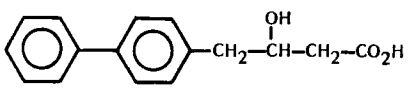

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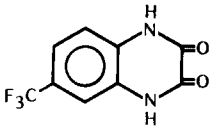
Comparative studies with acetaminophen indicate that metabolism in the hamster resembles more closely that in man than in mouse[117]. d-Limonene (**32**) is metabolized in rat, rabbit and hamster preferentially to C-1 carboxylic acid derivatives, whereas dog and man largely form the 8,9-diol, and the guinea pig metabolizes **32** by these two pathways to the same extent[98]. Compound **41** is substantially oxidized by rat and guinea pig, but not man, to biphenylacetic acid; this possibly explains the ineffectiveness of **41** in rheumatoid arthritis[118]. Sedative-hypnotic **42** is N-hydroxylated in the cat, a more sensitive species to the pharmacodynamic effects of this drug, but not in the rat[119]. Chronic methadone administration suppresses methadone N-demethylase in guinea pigs and rats but induces the enzyme in mice[120]. Finally, the importance of drug evaluation in both sexes is underscored by studies which showed that male but not female rats N-demethylated meptazinol (**43**) to a metabolite with increased biological activity[121,122].



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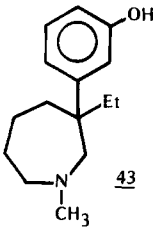

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Section V - Topics in Biology

Editor: T.Y. Shen, Merck & Co., Rahway, New Jersey

Section Editorial

Toward the goal of rational therapy a better understanding of the pathophysiological conditions, preferably at the biochemical or molecular level, is essential. The selection of topics for this section during the past three years more or less reflects this belief. The phenomenal advances in biomembrane research in recent years have not only provided some plausible explanations of the mode of action of many drugs but also suggested new directions for medicinal chemists to search for novel therapeutic agents. As biomembranes are not individual entities, like t-RNA or prostaglandin cyclooxygenase, but a dynamic organization of chemically distinct yet functionally interdependant components of ionic groups, saccharides, lipids, proteins and their conjugates, they are likely susceptible to regulation by a variety of chemical structures. In addition to the traditional antimetabolites, ligand analogs, and active site inhibitors, considering the predominance of physical processes and conformational changes involved in the function of membrane receptors, enzymes, transport and communication mechanisms, "allosteric" inhibitors should be particularly attractive as membrane regulators. The extensive interest in the chemical and physical characterizations of natural and artificial vesicles may also facilitate the synthetic study of these often elusive allosteric inhibitors. To illustrate the state of our knowledge, the two chapters on the chemical and biochemical characteristics of membrane receptors bring us up to date on some current concepts and experimental findings.

Continuing our survey of important but largely unexplored areas of drug research, it is encouraging to see the recent progress in muscular dystrophy, a major disease still without adequate remedies. Hopefully further advances in this area will improve the feasibility of developing chemotherapeutic agents. Following the recent breakthrough in Vitamin D biochemistry, a new chapter on metabolic bone disease is also presented.

With the growing concern about the toxicity of new chemicals, early safety assessment of product candidates or even potential "leads" to avoid costly investment in time and effort on potentially toxic compounds, has become a common practice. The degree or probability of carcinogenecity, mutagenecity and teratogenecity of chemicals are controversial legal and medical issues at times. To provide some perspective, a collaborative discussion by three authors on the nature and significance of some current laboratory procedures widely used in safety assessment is offered. It is certainly encouraging to witness the beginning of a new trend to rationalize the complex toxicity problem in terms of biochemical mechanisms.

Section V - Topics in Biology

Editor: T. Y. Shen, Merck & Co., Inc., Rahway, New Jersey 07065

Chapter 22. Relationships in the Structure and Function of Cell Surface Receptors for Glycoprotein Hormones, Bacterial Toxins, and Interferon

Leonard D. Kohn, Section on Biochemistry of Cell Regulation, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Introduction - Recent studies indicate that there is a structural and functional relationship in the mechanism by which glycoprotein hormones [thyrotropin (TSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), and follicle-stimulating hormone (FSH)], certain bacterial toxins (cholera and tetanus, for example), and the antiviral protective agent, interferon, transmit their message through the cell membrane. Thus, in each case these agents appear to be composed of two subunits or regions in their molecular structure, i.e., the α and β subunits of the glycoprotein hormones or the A and B proteins of cholera toxin. One subunit or region, the β subunit or B protein of the hormones and cholera toxin, for example, interacts with a receptor containing a ganglioside or ganglioside-like molecule whose oligosaccharide determinant causes a specific conformational change in the hormone molecule; interaction with a receptor containing the wrong oligosaccharide moiety will not cause this conformational change and will therefore not allow further message transmission. As a consequence of the initial interaction of the first subunit, as a consequence of the second subunit translocation, or as a consequence of both events together, a change in membrane state results. The change in membrane state expresses itself as a different exposure of cell surface determinants, including those important as receptors for other effectors of cell function and as a change in the electrochemical gradient across the membrane. Finally, as a consequence of some or all of these preceding events, changes in cyclic AMP levels, which are effected by modulating the activity of the adenylate cyclase system, transmit the effector message to the translational and transcriptional machinery of the cell.[1]

I. The Role of Gangliosides in the Thyrotropin (TSH) Receptor - Gangliosides are a family of glycolipids whose nomenclature is derived from the length of the oligosaccharide core structure (G_{M1}, G_{M2}, G_{M3}) as well as the number and location of sialic acid residues within the oligosaccharide moiety, i.e., mono-, di-, and trisialic acid derivatives (G_M, G_D, and G_T, respectively) are further defined isomerically (G_{D1a}, G_{D1b}, G_{T1}) on the basis of the galactose residue to which the sialic acid residues are linked.[2] The ganglioside G_{M1} is not only a component of the specific receptor for cholera toxin,[3] its interaction with toxin is presumed to initiate a sequence of events resulting (i) in the dissociation of the A from the B protein of the toxin and (ii) in the formation of an activated

A_1 subunit which translocates within the cell membrane and activates the adenylate cyclase system.

Recent studies[4-10] have shown that the gangliosides G_{D1b} and G_{T1} are potent inhibitors of TSH binding to its specific membrane receptor; that this inhibition is caused by an interaction of these gangliosides with the hormone rather than the membrane; that the inhibition is hormone-specific; and, most important, that these gangliosides appear to impose on the TSH molecule a conformation distinct from that resulting from the interaction of TSH with gangliosides which are poor inhibitors of TSH binding, i.e., G_{D1a}, the structural isomer of G_{D1b}. These results led to the suggestion that TSH had a receptor structure similar to that of cholera toxin and that both effectors were analogous in their message transmission process.[4-10] Specifically, the following was suggested: the β subunit of TSH interacts with receptor containing a ganglioside as a functional component; the ganglioside confers hormone or toxin specificity through its oligosaccharide determinant; the "correct" oligosaccharide determinant causes a unique conformation in its particular effector which is necessary for the α subunit to translocate within the membrane; the α subunit of TSH, like the A protein of cholera toxin, carries information on its primary sequence necessary for the stimulation of the adenylate cyclase system; and, last, cholera toxin and TSH might not only have a common membrane component with which they interact, but differences in such interactions might explain the toxicity of one and the regulatory role of the other.

Among the experimental evidence which has accumulated to support these predictions are the following: Thyroid plasma membranes are not only rich in gangliosides with the chromatographic characteristics of G_{D1b}, G_{T1}, and G_{M1},[4] but also in higher order ganglioside which (i) are even more potent inhibitors of TSH binding and (ii) do not appear to be present in the brain.[10] A TSH receptor defect in a rat thyroid tumor has been correlated with an alteration in the ganglioside content of the membranes of this tumor and with a defect in the biosynthetic enzymes concerned with the synthesis of the higher order gangliosides implicated as components of the TSH receptor structure.[7] The ganglioside G_{M1} induces a conformational change in cholera toxin which cannot be duplicated by other gangliosides[6,11] and cholera toxin interacts with thyroid plasma membranes, these interactions affecting the ability of TSH both to bind to the membranes and to stimulate the adenylate cyclase activity of the membranes.[6,9]

Regarding the adenylate cyclase stimulation, it is to be noted that NAD significantly enhances the ability of cholera toxin to stimulate adenylate cyclase activity in thyroid plasma membranes, whereas it inhibits the ability of TSH to stimulate adenylate cyclase activity in these same thyroid membranes.[6] This finding suggests that cholera toxin and TSH diverge in the molecular mechanism of adenylate cyclase stimulation and that there exist intermediate steps (involving NAD) between receptor recognition and adenylate cyclase stimulation.[3,6,12] The observation that cholera toxin has the ability to hydrolyze NAD in a manner resembling diphtheria toxin is an expecially intriguing finding in this area.[11,13,14]

Last, but far from least, support for the schema comes from the fact that sequence homologies have been demonstrated to exist between cholera toxin, TSH, and the other glycoprotein hormones[4,15] (Fig. 1A). Regarding the homology between the B protein of cholera toxin and the β subunits of the TSH-LH-hCG-FSH superfamily, it is especially notable (i) that the CAGY region of the glycoprotein hormones represents an important homology which has been highly preserved despite structural and functional differences which occurred in their evolution of progression to specialized target organ functions;[16] (ii) that this homology exists on that portion of the glycoprotein or toxin molecules, the β subunit or the B protein, which carries the primary determinants for receptor binding; and (iii) that the β subunits of the glycoprotein hormones determine their target organ specificity. This region of sequence similarity is thus an obvious candidate for the "active site" concerned with binding to receptors on the cell membrane.

Support for this interpretation is offered by two recent observations.[17] First, a synthetic decapeptide, including the CAGY sequence and the 3 residues on either side in the TSH sequence, will inhibit TSH binding and stimulate cholera toxin binding, i.e., have an effect similar to TSH itself. Second, trypsin, a protease with a CAGY sequence, can inhibit TSH binding to the intact and solubilized TSH receptor without destroying TSH receptor activity.

In regard to the sequence homology noted between the A protein of cholera toxin and α subunit of the glycoprotein hormones[5] (Fig. 1), this region is startlingly similar in sequence to the nonapeptide neurohypophyseal hormones, oxytocin and vasopressin,[9] i.e., to nonapeptides whose action also involves adenylate cyclase stimulation. Within this framework, it is to be noted that a 1,400 molecular weight fragment of the A_1 protein of cholera toxin, i.e., a 10- or 11-residue sequence, may be sufficient to cause the adenylate cyclase changes correlated with the action of this agent.[18]

II. A Change in the State of the Membrane Results from the Interaction of TSH or Cholera Toxin with Its Specific Receptors - A most important experiment concerning the cholera toxin-TSH interaction with thyroid plasma membranes came from the attempt to show by direct biochemical means that G_{M1} is the cholera toxin receptor on thyroid plasma membranes.[6] Thyroid membranes incubated with galactose oxidase, followed by exposure to sodium borotritide, should have tritium incorporated into ganglioside residues if these residues are available to the enzyme. When such an experiment was performed, the major labeled ganglioside of thyroid membranes was G_{M1}. As predicted by the hypothesis that G_{M1} is involved in the cholera toxin receptor structure, the same experiment performed on membranes pretreated with cholera toxin showed that the G_{M1} residues were not labeled with tritium. Most important, however, was the observation that pretreatment with cholera toxin caused an enhancement of the labeling of other glycolipids contained in the membrane which chromatographed in the region of G_{D1b}, G_{T1}, G_{M2}, and G_{M3}, i.e., caused an exposure of surface determinants important as receptors for other effectors of cell function.[6]

This last observation was of special interest in the light of the data which showed that low concentrations of cholera toxin enhanced TSH binding to thyroid plasma membranes and that there was a cooperative relationship between ganglioside or ganglioside-like receptors for the different effectors, TSH and cholera toxin. Since analogous data have been obtained when TSH interacts with the cell membrane,[9] it appears that a change in the state of the membrane is a general feature of the events immediately following the receptor-effector interaction.

III. The Role of Ganglioside or Ganglioside-Like Compounds in the Structure and Function of Receptors for Other Glycoprotein Hormones - The sequence homologies between LH, hCG, FSH, TSH, and cholera toxin (Fig. 1) raised the obvious possibility that all the glycoprotein hormones would have a similar receptor structure and a similar mechanism of receptor interaction. Each target organ would thus have as its receptor a ganglioside or ganglioside-like structure with a specific and unique carbohydrate sequence, i.e., one which was different from that on other target organs. The interaction of the appropriate hormone with this specific oligosaccharide would result in a unique conformational shift such that the α subunit would be placed in the position favored for membrane translocation and adenylate cyclase activation in that particular target tissue. Interaction with the wrong hormone would result in a different conformation, an unfavorable position for membrane translocation, and no transmission of its "message" to the cell membrane. A recent study of ganglioside inhibition of hCG binding to testes membranes provides preliminary support for a portion of this hypothesis in that the data show that the oligosaccharide moiety of the hCG receptor structure is distinct from the oligosaccharide moiety on the TSH receptor,[19] i.e., the most effective inhibitors of hCG binding, have a terminal sialic acid on the oligosaccharide chain, whereas the most effective inhibitors of TSH binding have a disialyl group linked to the internal galactose residue. Studies with LH are consistent with these conclusions in that gangliosides also inhibit LH binding to testes membranes,[20] the ganglioside specificity is distinct from that of both hCG and TSH,[20] and the different gangliosides impose distinct conformational changes on hCG and LH which can be correlated with the inhibition data.[19,20]

IV. Similarities in the Structure and Function of Receptors for Cholera Toxin and the Glycoprotein Hormones Extend to Interferon - Evidence has accumulated which suggests that the binding of interferon to specific cell surface receptors is necessary for the development of its antiviral action and that these receptors, like the glycoprotein receptors above, contain gangliosides or ganglioside-like structures whose oligosaccharide moiety is a critical feature of the receptor structure.[21-29] In this regard, experiments have shown that cholera toxin and TSH inhibit the establishment of the antiviral state by interferon;[24,25] these experiments were based on the hypothesis that cholera toxin or TSH might directly or indirectly interfere with the gangliosides or ganglioside-like oligosaccharide structures believed to be important for interferon's interaction with its cell membrane receptors. Direct *in vitro* binding experiments with cholera toxin and TSH[25] indicate that this notion is valid and establish that the antiviral action of interferon is a membrane-initiated event.

FIG. 1. Top: Sequences of the amino terminal 42 residues of the B protein of cholera toxin and of portions of the β subunits of TSH, LH, hCG, and FSH, defined by positions from the amino terminal residue of each molecule. The residue symbols corresponding to the standard residue abbreviations are as follows: A, ala; P, pro; D, asp; T, thr; S, ser; L, leu; I, ile; V, val; E, glu; G, gly; H, his; C, cys; N, asn; M, met; Q, gln; Z, glx; B, asx; R, arg; F, phe; Y, tyr; K, lys; W, trp; X, undetermined. Middle: The sequences of the amino terminal portions of the α subunits of TSH, LH, hCG, and FSH aligned to show an analogy with a fragment of the A_1 protein of cholera toxin, the cholera toxin subunit known to be responsible for adenylate cyclase activation. The sequence of cholera toxin used included the 42 amino terminal residues of the B protein as reported by Kurosky et al.[15,60] and the A protein sequences recently reported by Mendez et al.[61] All other sequences were from the tapes of the "Atlas of Protein Sequence and Structure,"[16] updated to December 5, 1975. Bottom: Comparison of the amino acid sequence of the neurohypophyseal nonapeptide hormones, oxytocin and vasopressin, with the amino acid sequence of a portion of the α subunits of the glycoprotein hormones. Sequence data are derived from the "Atlas of Protein Sequence and Structure."[16]

	Position		Position
Cholera toxin (partial fragment)	1	T P Q N I T D L C A E Y H N T Q I H T L N N K I F S Y T E S L A G K R E M A I I T F	42
TSH β chain (bovine)	19	C L T I N T T V C A G Y C M T R B V B G K L F L I P K Y A L S Q D V C T Y R D F M Y K	60
LH β (bovine)	26	C I T F T T S I C A G Y C P S M K R V L P V I L I P P M P E R V C T Y H E L R F A S V	67
hCG β chain (human)	26	C I T V N I T I I C A G Y C P T M T R V L Q G V L P A L P Z L V C N Y R D V R F E S I	67
FSH β chain (human)	24	C L T I N T T W C A G Y C Y T R D L V Y K D P A K P R I Q K T C T F K E L V Y E T V	65

	Position		Position
Cholera toxin (partial fragment)		Z Z P H I H A P G G C P B A P R	
TSH α chain (bovine)	1	F P D G E F T M Z G C P Z C K L K E N K Y F S K P B	26
LH α chain (bovine)	1	F P B G Z F T M Z G C P Z C K L K Z B K Y F S K P B	26
hCG α chain (human)	1	A P B V Z B C P Z C T L Z Z B P F F S Z P G	22
FSH α chain (human)	1	A P D V E D C P E C T L Q E N P F F S Q P G	22

	Position		Position
Oxytocin	1	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly	9
Arginine vasotocin	1	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly	9
Arginine vasopressin	1	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly	9
Lysine vasopressin	1	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly	9
hCG (human)	1	Ala-Pro-Asx-Val-Glx-Asn-Cys-Pro-Glx-Cys-Thr-Leu	12
FSH (human)	1	Ala-Pro-Asp-Val-Glu-Asn-Cys-Pro-Glu-Cys-Thr-Leu	12
TSH (bovine)	1	Phe-Pro-Asp-Gly-Glu-Phe-Thr-Met-Glx-Gly-Cys-Pro-Glx-Cys-Lys-Leu	16
LH (bovine)	1	Phe-Pro-Asx-Gly-Glx-Phe-Thr-Met-Glx-Gly-Cys-Pro-Glx-Cys-Lys-Leu	16

Experiments using TSH or cholera toxin as probes of the mechanism of interferon action also indicate the following: (i) The initial binding of interferon to a ganglioside or ganglioside-like receptor is insufficient to establish the antiviral state, i.e., human KB-3 cells which are unresponsive to either mouse or human interferon can bind both.[25] (ii) The receptor for interferon has both a glycoprotein and a ganglioside as its structural components.[28,29] (iii) Immediately following the interaction of interferon with this receptor structure, there is a change in membrane state analogous to that seen when cholera toxin and TSH interact with thyroid membranes; cells nonresponsive to interferon but able to bind interferon (human KB-3 cells) do not have the same change.[28,29] (iv) The failure of human KB-3 cells to transmit the interferon message to the cell machinery, despite the ability of interferon to bind to the cell surface of the cells, correlates with a near absence of membrane gangliosides, especially G_{M2} and G_{M3}, which are present in high levels in membranes of sensitive mouse L cells.[28,29] (v) Interferon stimulates adenylate cyclase activity in sensitive cells, maximal stimulation preceding the establishment of the antiviral state.[29,30] (vi) Interferon induces changes in the electrochemical gradient across the cell membrane; this effect may be related to the ability of ouabain to inhibit interferon action (see below).

V. Similarities in the Structure and Function of Receptors for Cholera Toxin and the Glycoprotein Hormones Extend to Other Bacterial Toxins and This Relationship Has Pathophysiological Significance - The above observations raised the possibility that bacterial products other than cholera toxin with a known tropism for gangliosides such as tetanus toxin and Escherichia coli enterotoxin might interact with thyroid plasma membranes, and even with the TSH receptor on the membranes. In this regard, tetanus toxin is of particular interest, since the specificity of the ganglioside-tetanus interaction ($G_{D1b} = G_{T1} > G_{M1} > G_{D1a} > G_{M2}$)[31] is similar to the specificity of the ganglioside-TSH interaction and since the clinical presentation of tetanus includes a syndrome which closely resembles thyroid storm.[32-34]

When recently studied, tetanus toxin was shown to interact with thyroid plasma membranes.[35-38] More important, the properties of this interaction were shown to closely resemble the properties of the TSH interaction with TSH receptors on these membranes; the interaction of tetanus toxin with thyroid membranes exhibited several characteristics of its interaction with neural tissue; tetanus toxin would not bind to membranes of a thyroid tumor with a TSH receptor defect; and tetanus toxin could stimulate thyroid hyperfunction in mice subjected to conditions known to precipitate thyroid storm.

The ability of tetanus toxin to interact with thyroid membranes and with TSH receptors on these membranes, as suggested above, has several significant implications and applications. An obvious clinical implication is that the syndrome of sympathetic overactivity which can appear in tetanus is contributed to by the ability of the toxin to induce thyroid hyperfunction. This syndrome would thus be analogous in its etiological relationship to the thyroid as is thyroid storm or as is the hypersensitivity to

catecholamines demonstrable in thyrotoxic patients. A second implication is that tetanus toxin will have a receptor structure and mechanism of effecting cell processes analogous to cholera toxin, the glycoprotein hormones, and interferon. It is thus no surprise that tetanus toxin has two different functional subunits, only one of which is responsible for its interaction with gangliosides.[39-41] It can be presumed that tetanus toxin will have the common primary sequences within these two subunit or structural components already demonstrated for cholera toxin and the glycoprotein hormones; that tetanus toxin will undergo a specific conformational change upon interacting with the receptor; and that the tetanus toxin action will also require or induce a change in membrane state or a reordering of membrane components. In short, it seems reasonable to presume that tetanus toxin interactions with the TSH receptor on thyroid plasma membranes are analogous to its interactions with neural tissue membranes and that studies which compare the tetanus toxin effects on thyroid membranes with those of TSH will clarify both the mechanism by which tetanus exerts its neurotoxic effects and TSH its thyroid-stimulating effects.[35-38]

VI. The Mechanism by Which Glycoprotein Hormones, Interferon, and the Bacterial Toxins Effect Cell Changes - Current views regarding the mechanism of action of TSH and other glycoprotein hormones invoke alterations in the concentration of cyclic AMP as the second event in the transfer of information from the hormones to the appropriate cell.[1] That is, the information carried by the hormone is purportedly translated into a "second message" by means of a change in intracellular cyclic AMP concentration.

One implication of the schema outlined above is that the increase in intracellular cyclic AMP may not necessarily represent a second message *per se*, but rather an event which is sequentially or simultaneously coupled to a primary effect of these agents on the membrane. In other words, the common denominator of all of the effectors may be an alteration in the membrane which leads to generalized effects on cellular metabolism, one of which may be an increase in the concentration of intracellular cyclic AMP. Viewed in this manner, it is clear that one means of accomplishing this end could be through alterations in electrochemical ion gradients across the cell membrane. In this regard, the following observations are notable: (i) The primary effect of tetanus toxin is an alteration in neural transmission;[42] (ii) cholera toxin causes a dramatic loss of electrolytes and water through the intestinal epithelium;[43] (iii) TSH increases the rate and extent of iodide uptake by thyroid cells;[44] (iv) oubain, the classic inhibitor of sodium, potassium-stimulated ATPase, blocks the antiviral action of interferon;[45] and (v) electrochemical ion gradients play a primary role in active transport and metabolism in prokaryotes, eukaryotes, and many subcellular organelles.[46]

With these items in mind, a recent development is the demonstration that TSH causes an increase in the uptake of the lipophilic cation triphenylmethylphosphonium ($TPMP^+$) when added to cultured thyroid cells or to membrane vesicles derived from these cells under appropriate conditions and that this effect is dependent upon a specific interaction of TSH with its receptor at the cell surface.[47] Since uptake of $TPMP^+$ and other lipophilic

cations has been shown to reflect the presence of an electrical potential across the membrane (interior negative), it is tempting to speculate that one of the primary effects of TSH is to hyperpolarize the thyroid cell membrane. Experiments with thyroid slices utilizing a direct electrophysiological approach[48] are consistent with this interpretation, i.e., TSH at low concentrations resulted in increased voltage measurements.

Since the effect of TSH on $TPMP^+$ uptake precedes the effect of the hormone on adenylate cyclase activity,[47] the possibility exists that a primary mode of action of each of these effectors is to alter electrochemical ion gradients across the cell membrane. This concept is attractive in that it would serve to explain certain other findings. Thus, hCG causes changes in adrenal cell ion transport which not only precede adenylate cyclase stimulation, but occur at concentrations of the effector which have minimal effects on cyclase activity;[49] cholera toxin and its B protein induce alterations in the permeability of liposomes reconstituted with "receptor" ganglioside in the absence of adenylate cyclase.[50,51]

VII. Structural Features of the Receptor - It has been reported previously that TSH receptors on bovine thyroid plasma membranes can be solubilized with lithium diiodosalicylate (LIS) and that tryptic digestion of the solubilized receptor preparation yields a 24,000 molecular weight receptor fragment which retains specific TSH binding activity.[52,53] Analysis of a purified preparation of this receptor fragment indicated that it was a glycoprotein containing 30% carbohydrate and 10% sialic acid by weight.[52,53] The above discussion indicates that gangliosides may be important components of the TSH receptor and that the role of gangliosides in trasmitting the hormonal message to the cell machinery is analogous to their role in transmitting the message of cholera toxin to cells exposed to this bacterial product.

Recent studies[54] have shown that the two components of thyroid plasma membranes known to interact with TSH, the glycoprotein with specific TSH binding activity, and the gangliosides of the thyroid membranes, segregate differently when membranes are solubilized with lithium diiodosalicylate. More important, the possibility raised in this study was that the ^{125}I-TSH binding activity of the intact plasma membrane is contributed to by both the glycoprotein and the ganglioside components of the membrane. Thus, the salt sensitivity of the intact plasma membrane receptor as well as its high degree of hormonal specificity in regard to other glycoprotein hormones (LH and hCG, for example) appears to reflect the ganglioside-TSH interaction. The conclusion concerning the salt sensitivity of the TSH-ganglioside interaction has been confirmed in direct ^{125}I-TSH binding experiments using ganglioside-containing liposomes.[55] The high degree of hormonal specificity is evident in the close correlation of inhibition of binding with the conformational changes effected in the different hormones by the different gangliosides (see above) and is in contrast to the loss of specificity for LH and hCG exhibited by the glycoprotein receptor fragment.[52,53,55]

In sum, these data coupled with previous observations concerning (i) the protease sensitivity of TSH binding and cyclase stimulation in cultured

thyroid cells[56] and (ii) the loss of TSH binding and cyclase stimulation in a thyroid tumor whose membranes are deficient in their ganglioside content,[7] suggest that each component contributes to the function of the intact plasma membrane receptor and that the functional transmission of the TSH message to the thyroid cell machinery requires the presence of both a glycoprotein and glycolipid component in the TSH receptor structure.[54]

It is not clear how the two components function in the binding of TSH and in the transmission of the TSH message to the cell machinery. The binding of TSH may involve both components simultaneously or, more likely at this moment, there may be a sequential interaction with one (the glycoprotein) and subsequent transfer or association with the second (the ganglioside), i.e., one can be the primary interaction site (the glycoprotein) and the other (the ganglioside) can be the obligatory discriminator and coupler to other cell processes.[54]

The existence of both glycolipid and glycoprotein membrane components with receptor specificity has recently been noted in studies of cryoglobulin interactions with cell membranes.[57] It is also evident in the fact that there are both glycolipid and glycoprotein components of the cell with blood group specificity.[58] The latter precedent is especially pertinent since it suggests that the glycolipid and glycoprotein components of the TSH receptor may contain analogous oligosaccharide structures. The existence of membrane glycoproteins able to specifically bind the effector is not unique to TSH; thus, they have been shown for hCG[59] and interferon[28,29] (in human KB-3 cells).

Summary - The details of the schema outlined in the introduction of this review are not defined for each individual agent; however, the relationships are increasingly clear. Although the implications to the pathophysiology of Graves' disease and the mechanism of action of cholera and tetanus toxin are already obvious, it is clear they will extend to other problems. Thus, the observation that contraceptive programs involving the development of antibodies to hCG have also caused significant levels of antibodies to tetanus in some patients, may be the tip of an iceberg, i.e., the implications will extend even to a "normal" disease such as pregnancy. In regard to medicinal chemistry, these relationships clearly imply caution. Yet it is hoped they will hold new opportunities to the development of peptide and carbohydrate analogs which will either block the action of cell membrane effectors which can cause a disease (cholera or tetanus toxin) or mimic the action of effectors regulating body functions (glycoprotein hormones) and blocking viral infestation of the cell (interferon).

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Chapter 23. Mineral Metabolism and Metabolic Bone Disease

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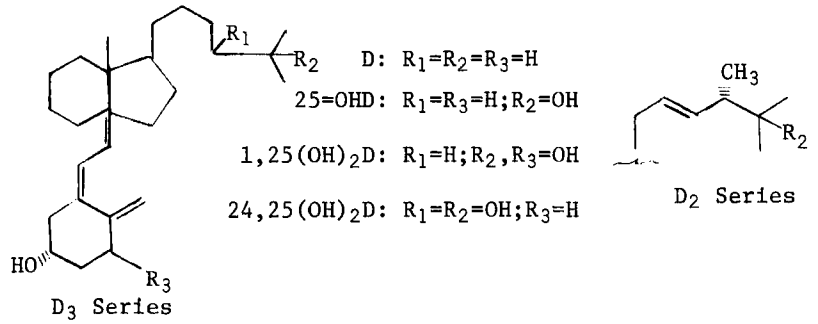
Introduction - Rapid progress is being made toward a better understanding of mineral metabolism. Advances have been made possible by breakthroughs in several divergent areas of research. Among these are the discovery of the active metabolites of vitamin D (reviewed in this series, Vol. 10, Chapter 30), elucidation of the biochemistry and endocrinology of the calcitropic hormones, the devising of a surgical technique which permits obtaining serial bone biopsies, and histomorphometric methods which allow reliable quantitative assessment of human bone. Biologically active compounds are available for study and the rationale for the preparation of others is forthcoming. The opportunities for the medicinal chemist to contribute to the solution of medical problems involving derangements in mineral metabolism are, therefore, greatly enhanced. The objectives of this review are to outline briefly some new concepts in the field and to provide a key to the pertinent literature.

Since the 1930s it has been known that vitamin D is important for normal growth and skeletal development of animals and man. It was recognized that there is a vitamin D requirement for absorption of calcium and phosphorus from the intestinal tract and it was appreciated that maintenance of serum calcium levels within rather narrow concentration limits was critical for normal function and even life itself. But it was not realized until much later that the skeleton plays a vital role in calcium and phosphorus homeostasis and is a dynamic tissue metabolically. The recent flood of knowledge on the biochemistry & endocrinology of vitamin D metabolites[1-3] parathyroid hormone (PTH),[4,6] calcitonin (CT),[6,7] the prostaglandins (PG),[7-9] and the cyclic nucleotides[10,11] has enabled the biochemist and cell physiologist to focus attention on the precise mechanisms involved in mineral homeostasis.[12] Their findings provide the basis for cautious optimism regarding the potential for the development of definitive diagnoses and effective treatments for many forms of metabolic bone disease.

Vitamin D Endocrinology - The significance of the active metabolites of vitamin D was aptly expressed by Kodicek[13] in 1974 when he wrote, "It was first descriptive, it reached then a physiological niveau, and eventually deepened into basic insights at molecular level and suddenly opened new practical vistas in medicine." Vitamin D must first be metabolically altered before it functions. The functional metabolites[14] are formed in organs other than their sites of action. Their rates of synthesis and secretion are feedback regulated and this regulation is effected at least in part by PTH, possibly by CT, other hormones and metabolic regulators and by calcium and phosphorus levels in certain tissues. The active metabolites play important roles in the control of calcium and phosphorus movement from both intestine and bone. They function as major humoral substances in the regulation of serum calcium concentration.[15] It is clear now that vitamin D is not simply a catalytic vitamin substance, but one



whose metabolism is dynamically interrelated with its function in an endocrine system.[15] With current knowledge, albeit incomplete, it is possible to speculate with some confidence on how derangements in vitamin D metabolism may be involved in the etiology of certain types of metabolic bone disease. Most of the known metabolites of vitamin D and many analogs and structural variations have been synthesized.[14-16] The clinical evaluation of these compounds for safety and efficacy is now progressing rapidly.

Biochemical Measurements - Any meaningful evaluation of an experimental drug in the treatment of metabolic bone disease requires dependable methods for measurement of the pertinent minerals and hormones. Atomic absorption methods[17] meet the requirement for accurate determination of total serum calcium and magnesium. Since PTH is more responsive to ionized calcium (Ca++) than to total serum calcium,[18,19] it is fortunate that methods for this measurement have become available recently in the form of specific electrodes.[20] Very satisfactory isotopic methods[21,22] for measuring intestinal calcium absorption are available. Total inorganic phosphorus is usually determined by the method of Fiske and SubbaRow.[23,24] Radioimmunoassays (RIAs) reasonably specific for C-terminal and N-terminal portions of human PTH have been developed by several groups.[25-27] Practical RIAs for human CT have been developed.[28,29] No successful RIA has been reported, but satisfactory competitive protein-binding radio assays and high pressure liquid chromatography assays have been devised for measurement of the ng/ml. levels of 25-OHD in serum.[30-32] Chromatographic separation of 25-OHD_2 and 25-OHD_3 has been reported[33] but separation is generally not required for most clinical studies. Measurement of the kidney metabolite, $1,25(OH)_2D_3$, is more difficult because of the pg/ml. concentrations encountered, but sensitive radio receptor assays have been devised[34,35] and used to measure serum levels in normal man and in various disease states.[36] A method specific for the determination of $24,25(OH)_2D_3$ was reported[37] recently and will undoubtedly be important for certain clinical evaluations. Since certain prostaglandins, particularly those of the E series, have osteolytic activity comparable to that of PTH, there are situations where measurement of these lipids is useful. RIAs for PGEs and their metabolites have been reported,[38] although most investigators have had difficulty preparing antisera with the required specificity. Both competitive binding assays[39] and RIA[40] have been developed for measurement of cAMP.

Histomorphometric Measurements - Determination of the status of the bone

tissue is vitally important for diagnosis and evaluation of changes induced by experimental treatment. This is accomplished best by direct examination of bone biopsies. Until recently the only surgical procedure for obtaining a bone specimen was the "open biopsy" for removal of a wedge from the iliac crest or a segment of a rib while the patient was under general anesthesia. These procedures were so traumatic and incapacitating for the patient that it was rarely possible to gain consent for a second biopsy. The essentially non-traumatic transilial biopsy technique of Bordier,[41] which is performed with a trephine and local anesthesia, produces a cylindrical bone sample or core with dimensions of 8-12mm in length and 5-8mm in diameter. This method has made repeated biopsies an entirely feasible procedure for diagnosis and drug evaluation studies.

Coincident with this new technique for procurement of human bone biopsies was the development of quantitive methods of bone analysis.[12] These methods include histochemical analysis of both decalcified and undecalcified[42,43] bone sections, microradiography,[44] tetracycline labeling[45] and autoradiography.[42] The latter two techniques require administration of a tetracycline antibiotic or isotopic tracer prior to procurement of the biopsy. Undecalcified thin sections, prepared with the use of a Jung microtome after the bone core is fixed, dehydrated and embedded in methacrylate,[45] are analyzed by intersect and point count methods[46,47] which permit three-dimensional assessment.[48,49] Tetracycline antibiotics deposit *in vivo* in sites of bone formation constituting markers which can be studied in undecalcified sections by fluorescence microscopy.[45,47] This represents the safest and best tissue time marker for microscopic measurement of bone formation dynamics.

Largely through the application of these new techniques some basic concepts of bone physiology and metabolism are beginning to be appreciated. Normal human bone undergoes four distinct processes: growth, modeling, repair and remodeling.[50] From fetal development until some time after puberty, growth and modeling are the predominant processes during which bones grow both in length and in width. The modeling process is responsible for insuring proper shape and proportion of the growing skeleton. After maximum growth is achieved, the modeling process ceases, but the skeleton remains metabolically active and undergoes a continuous process of remodeling through resorption and formation. The process of repair is operative at all stages of life and comes into play whenever a fracture occurs in any member of the skeleton. This process appears to be quite independent of the other two and is usually operative even when modeling or remodeling is defective.

Metabolic bone disease in adults nearly always represents some derangement in the remodeling process. This point must be considered in choosing experimental animal models for human disease. For example, the rat cannot serve as an adequate model for the study of human osteoporosis because it never completes the modeling phase in its life cycle. Osteoporosis in man is a disease involving derangement of the remodeling process. Metabolic bone diseases in children are usually more complicated because defects in both the modeling and remodeling processes may be

involved.

The remodeling process, involving continuous resorption of both the mineralized portion of bone and the organic matrix, is coupled with the formation process and must precede it. In other words, in normal bone, formation cannot occur until resorption has taken place. In recent years much has been learned about this microscopic remodeling system which operates continuously in the entire skeleton.[12] Bone cells known as osteoclasts are responsible for the resorption process. Formation and mineralization are initiated by cells known as osteoblasts. Modern histomorphometric techniques applied to the cells and surfaces of serial bone biopsies enable researchers to detect and quantitate malfunctions underlying the pathology in various types of metabolic bone disease. With this capability the utility of these techniques in diagnosis and evaluation of treatment are obvious.

Non-Invasive Methods of Skeletal Analysis - While information provided by examination of human bone biopsies is invaluable, it is well recognized that the approach has serious limitations. Fortunately, much progress has been made in the development of supplemental non-invasive techniques for assessment of bone mineral content in health and disease. Research initiated years ago by physicists has yielded results which are being widely utilized now by physicians in the study and treatment of metabolic bone disease. The roentgenogram or x-ray was the first of the non-invasive methods in this category, has been used for decades, will never be completely replaced, but suffers from a number of shortcomings. Lack of sensitivity is a serious limitation especially for use in detecting and quantitating skeletal demineralization. Using standard x-ray procedures, it is difficult to detect demineralization until a 30-40% change has taken place.[51] The lack of mono-chromicity and dangers from radiation exposure represent further limitations. However, some modern specialized techniques for quantitating changes in metacarpal and phalangeal bones of the hand,[52] and others for detection of ectopic calcification[53] are extremely useful.

The photon absorptiometry procedures pioneered by Cameron and Sorenson[54,55] are finding widespread use.[56-59] This approach provides a sensitive method for measuring both cortical and trabecular bone mass in the human radius and ulna. Bone mineral mass or content is determined by measuring the photon energy absorbed by bone from a monochromatic gamma-ray source, usually ^{125}I. Standardized instruments are commercially available and are being used in many hospital and research centers. Modifications of the general method are being developed to permit bone mineral content measurements of other parts of the skeleton.[59]

Total body neutron activation analysis and whole body counting[60] permit the determination of the absolute quantity of certain minerals (including calcium and phosphorus) in the entire body with great accuracy and reproducibility. Since absolute measurements of the total body stores of such elements as calcium and potassium have been normalized for size, sex, age, and body habitus in individual patients, estimates of normal, increased and decreased stores of these elements are possible in a wide

variety of metabolic conditions. Unfortunately, there are very few centers equipped to carry out such sophisticated measurements.

Derangements in mineral metabolism frequently lead to metastatic calcification of soft tissue. Bone-seeking radionuclides, such as ^{99m}Tc-labeled phosphate preparations, are proving particularly useful in detecting extra-skeletal calcification in situations where roentgenographic demonstration is lacking.[61]

Clinical Studies

a) Paget's Disease - This fairly rare disease is characterized by bizarre malfunctioning of the bone remodeling process in which both resorption and formation occur at grossly accelerated and unmatched rates.[12] Both CT and disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) have been used in the treatment of this painful and disabling malady. Combination therapy with human CT and EHDP[62] appears to offer the best treatment to date.

b) Hypoparathyroidism - This disorder is caused by a deficiency of PTH or an impairment of parathyroid functions leading to a state of hypocalcemia. Most cases result from surgical removal of parathyroid glands as the treatment for thyroid or parathyroid malignancy. Ideal treatment for surgical and idiopathic forms would be administration of human PTH. Since this is not available and because prolonged treatment with bovine PTH causes a refractory state due to antibody formation, some form of vitamin D is the only currently available treatment. Clinical experience indicates that pharmacologic doses of vitamin D,[12] $25\text{-}OHD_3$[63] or $1,25(OH)_2D_3$[63] can produce many of the effects of PTH in the intestines, kidney and bone to stimulate a rise in serum calcium although by somewhat different mechanisms.[12] Dihydrotachysterol and $1,25(OH)_2D_3$ offer the advantage of rapid onset of action in initiating treatment in acute post-surgical hypoparathyroidism while in chronic hypoparathyroidism treatment with $25\text{-}OHD_3$ induces good stability of plasma calcium.[64]

c) Renal Osteodystrophy - This term is used to describe the bone disease which accompanies renal insufficiency. Severity ranges from asymptomatic to complete disablement, although abnormality at the cellular level is readily detected even in insipient renal insufficiency.[65] The incidence of this disease has increased rapidly during the last 15 years as the result of ever increasing availability of dialysis facilities. The consequences of renal failure resulting from disruption of both the excretory and endocrine functions of the kidney include hyperphosphatemia, secondary hyperparathyroidism, hypermagnesemia and impaired intestinal absorption of calcium. Serum calcium levels are usually in the low normal range, but a significant number of these patients are hypocalcemic. Correction of hypocalcemia when it occurs does not necessarily cure the disease. Skeletal demineralization and muscular weakness are evident as the disease progresses. The classical skeletal lesions are osteomalacia (an excess of unmineralized bone matrix or osteoid) and osteitis fibrosa (fibrotic tissue invasion of the bone). The former is believed to be caused by a metabolic resistance to the actions of vitamin D and the latter to the influence of excess PTH.[50,66]

Recent studies on the treatment of renal osteodystrophy have concentrated on evaluation of the newly discovered metabolites of vitamin D. Because kidney enzymes are required for *in vivo* production of $1,25(OH)_2D_3$,[14] many investigators assumed logically that administration of this metabolite would solve the problem of vitamin D resistance. Results to date indicate that $1,25(OH)_2D_3$ and the analog, 1α-OHD_3, are remarkably potent in stimulation of intestinal calcium absorption, they lower elevated serum alkaline phosphatase and are of some benefit in control of secondary hyperparathyroidism.[67-72] Hypercalcemia has been encountered[68,70,71] and remineralization of depleted bones has not occurred to the extent anticipated.[68,69,72] Studies evaluating pharmacologic doses of 25-OHD_3 have produced encouraging preliminary results.[73,74] Some investigators conclude that the osteomalacia of renal failure is caused by a lack of 25-OHD_3 rather than a deficiency of $1,25(OH)_2D_3$ alone.[75] Very recently results reported on the use of $24,25(OH)_2D_3$ indicate that this metabolite, which is produced from 25-OHD_3 in the kidney and elsewhere in man is specifically active in remineralization.[76]

d) Vitamin D-Dependent Rickets - This form of metabolic bone disease is an inborn error of vitamin D metabolism due to a genetic defect in the 25-hydroxyvitamin D-1-hydroxylase, the renal enzyme responsible for conversion of 25-OHD_3 to $1,25(OH)_2D_3$.[77] It has been known for years that this rare disorder was manageable with massive doses of vitamin D and recent studies[78,79] have shown that 25-OHD_3 in doses of 100 to several hundred micrograms/day will provide complete clinical, biological and radiological recovery. Similar results have been reported[77] using ca 1 μg/day of $1,25(OH)_2D_3$.

e) Familial Hypophosphatemic Vitamin D-Resistant Rickets - There appear to be several types of hereditary vitamin D-resistant rickets (VDRR) associated with hypophosphatemia due to diminished tubular reabsorption of phosphate.[80] This defect may also involve reduced reabsorption of amino acids or glucose. Large doses of vitamin D may in some cases improve the rickets with variable effect on the tubular reabsorption defect.[81] Both 25-OHD_3 and $1,25(OH)_2D_3$ have been evaluated to some extent.[82-84] Neither compound is highly effective, but large doses of 25-OHD_3 have induced beneficial effects in about half of the cases investigated.

f) Anticonvulsant Osteomalacia - This term has been applied to the skeletal demineralization condition which appears to be induced by administration of anticonvulsant drugs, particularly dephenylhydantoin and phenobarbital.[85] Patients, usually epileptics, taking these drugs tend to have lower than normal serum 25-OHD levels and slight hypocalcemia. The incidence appears to be higher among mentally retarded patients[86] and varies from one location to another.[85] The disorder is believed to be caused by some abnormality in vitamin D metabolism induced by the action of the anticonvulsant drug on the liver, but the details are not clearly understood.[87] In many instances the problem has been reported to be minimized by administration of 1000-4000 I.U. of vitamin D per day. However, cases resistant to this treatment have been reported and these responded well to small doses of 25-OHD_3.[86] Clearly more research is required to solve this con-

troversial problem.

g) Steroid-Induced Osteopenia - Glucocorticoids and synthetic analogs are employed frequently as pharmacologic agents in the treatment of a wide variety of diseases for prolonged periods of time. One of the most serious complications in the use of these steroids is the development of a metabolic bone disease known as steroid-induced osteopenia or osteoporosis. The decrease in bone mass encountered in these situations is believed to depend upon two influences of the steroids: 1) an inhibitory effect on osteoblasts resulting in decreased bone formation and 2) stimulation of mild secondary hyperparathyroidism. The latter is thought to be related to the well established influence of steroid hormones to inhibit intestinal absorption of calcium.[88] Recent animal studies[89] showed that prednisolone administration caused partial inhibition of the action of $1,25(OH)_2D_3$ upon intestinal calcium transport by induction of an enzyme system which catalyzed conversion of $1,25(OH)_2D_3$ to a more polar, biologically-inactive metabolite. A preliminary report[90] on a clinical study evaluating $25\text{-}OHD_3$ in the treatment of steroid-induced osteopenia claimed that a dose of 40 μg/day for a mean treatment period of 11.3 months led to significant improvement in intestinal calcium absorption, normalization of PTH levels and a 20% increase in mean trabecular bone mass. These results were achieved with patients continuing on their steroid therapy. If these impressive results can be confirmed and expanded, this would represent a significant advance. An effective method for prevention and resolution of steroid-induced osteopenia is important not solely as a benefit for the large population threatened by or suffering from this disorder, but also because this malady is believed to represent a possible model for postmenopausal and senile osteoporosis. Any therapy shown to be effective in the management of steroid-induced osteopenia should be carefully evaluated as a treatment of other forms of osteoporosis.

h) Liver Disease & Malabsorption Syndromes - The introduction of the competitive protein-binding radioassay for the circulating metabolite, $25\text{-}OHD_3$, has permitted studies which correlated subnormal serum levels of $25\text{-}OHD_3$ with a variety of pathological conditions. In addition to patients receiving anticonvulsant drugs, it has been found that low levels of 25-OHD are common in patients with derangement of the excretory function of the liver,[31] particularly primary biliary cirrhosis[91] and biliary atresia,[92] patients with renal stones,[93] alcoholics,[93] ulcerative and granulomatous colitis,[94] regional enteritis,[94] and granulomatous ileocolitis.[94] In all of these conditions the finding of subnormal serum 25-OHD concentration is associated with a significant incidence of skeletal demineralization. In patients with various types of inflammatory bowel disease more profound skeletal defects occur in adolescents than in adults. Neonatal hypocalcemia is frequently associated with subnormal 25-OHD serum levels, especially in premature infants.[95] From work reported to date, it is clear that hypo-25-hydroxycholecalciferolemia is far from rare, but escaped detection until a suitable diagnostic test became available. Evaluation of treatment of these disorders with exogenous $25\text{-}OHD_3$ and $1,25(OH)_2D_3$ is just beginning.[91,96]

i) Hypercalcemia - The most common causes of acute hypercalcemia are hyperparathyroidism and neoplastic disease. In the latter case little is known of the mechanism of the hypercalcemia. Only in a few instances have tumors been shown to secrete PTH. Recently prostaglandins have been identified as mediators of hypercalcemia associated with certain human tumors.[97] Administration of PG-synthetase inhibitors, indomethacin and aspirin, caused reduction in the excretion of PGE metabolites and a concomitant fall in serum calcium. Organ cultures of human myeloma cells secrete an osteoclast-activating factor which is different from either PTH or PG.[98] The activity of another bone-resorbing factor, complement, is mediated by activation of the PG synthetase system in bone.[99] Current findings are consistent with mediation of hypercalcemia not only by PGE_2 but also by its endoperoxide or by thromboxane A_2.[98,100] Research on inhibitors of fatty acid cyclo-oxygenase as agents for treatment of patients with hypercalcemia of neoplasia offers attractive possibilities.

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Chapter 24. Detecting Mutagens - Correlation Between the Mutagenicity and Carcinogenicity of Chemicals

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The purpose of mutagenicity testing is two-fold. The first purpose is to determine whether a chemical substance has the capacity to cause alteration of genetic information with the potential to produce heritable genetic changes in man. The fundamental concern, therefore, is the risk of mutation to future generations as might result from a genetic alteration occurring within the germ cells of either a male or female.

The other purpose of mutagenicity testing relates not to germ cells but to somatic cells, where the concern is one of cancer. As will be described in later sections, there are compelling reasons which are both empirical and theoretical to believe that certain mutagenicity test systems have a capacity to identify chemical carcinogens with a high degree of accuracy. Recent attention paid to mutagenicity test systems by industry and Federal regulatory agencies has in large part related to this latter purpose. Clearly there exists a great need for a short-term, highly sensitive, and reliable prescreen for the identification of potential chemical carcinogens which at approximately one-hundredth the cost of lifetime studies in animals can effectively identify potential carcinogenic hazards. An understanding of mutation and the functioning of mutational test systems depends upon knowledge of the chemical nature and molecular organization of genetic material. The genetic material of all living organisms, with the exception of certain viruses, is composed of deoxyribonucleic acid (DNA). DNA is an enormously long polymer containing as many as 10^8 nucleotides linked by phosphodiester bonds.

The development of a good correlation between the mutagenic and carcinogenic properties of chemicals would suggest the possibility that a molecular event may be shared by the two phenomena. A theoretical basis for relating mutagenesis and carcinogenesis has been proposed by several investigators based on the concept that neoplasms arise from mutations in somatic cells.[3,82,83] This has long been a popular theory of cancer causation and there is increasing widespread belief among cancer workers that DNA damage is involved in the induction of cancer. It is known that cell regulation can be easily altered by mutation and that a heritable change in cell regulation is a characteristic property of a cancer cell. Other indirect evidence accumulated during the last several years has begun to implicate the role of mutations in chemically-induced malignant transformation.[84,85] However, mutation as an essential step in carcinogenesis remains to be clearly demonstrated, and at present, the relationship is mainly derived from recent experimental evidence.[2,55,3,82,83,86-92]

There are four main nitrogenous bases which appear in DNA--two of

them are purines (adenine and guanine) and the other two are pyrimidines (thymine and cytosine). In all living organisms, with the exception of certain viruses, DNA molecules occur in nature as paired strands in the form of a double-stranded alpha helix in which the hydrogen bonds of guanine and adenine interact specifically with cytosine and thymine respectively. The biological significance of this arrangement is that one strand of the DNA double helix can serve as the template for the synthesis of its opposite, or partner, strand. The base sequences encode the genetic information of the gene and the replication mechanism permits the propagation of the genetic material.

Were one of the nitrogenous bases within DNA altered (damaged) by chemical reaction with a mutagen, such damage could lead to mutation. For example, a chemical might interact with guanine moieties of DNA altering its hydrogen bonding properties so that instead of forming specific hydrogen bonds with cytosine pairing occurs with thymine. Another common example of chemical alteration of DNA is the loss of a nitrogenous base through hydrolysis of the beta glycosidic bond. Such alterations are referred to as point mutations because they may affect only a single or a few nucleotides within a gene consisting of hundreds or thousands of nucleotides. Point mutations are a well known cause of mutations which exist within human populations. Sickle cell anemia is an example of a point mutation that has been well characterized and has a profound effect upon mutant individuals. Other examples of point mutations or single-gene mutations include cystic fibrosis, hemophilia, and Tay-Sachs disease.[97]

Another major class of human mutations affects not the genes *per se* but chromosomes, the large units into which genes are organized. A single chromosome may contain as many as 10,000 genes. The core of a chromosome is believed to consist entirely of DNA and, therefore, its backbone is the phosphodiester. Chromosomal mutations include either chromosome breaks which would necessarily involve cleavage of the phosphodiester arrangements of the chromosomal segments (translocations, inversions) which are predicated on a combination of phosphodiester breaks and rejoinings of phosphodiester bonds. Many of the chromosomal mutations occurring in man involve the loss of the chromosome, which can result from either breakage of the phosphodiester bond, or a process referred to as nondisjunction, which does not, as far as is known, involve DNA *per se*. Other mutations result from duplication of part or all of the chromosome.

Mutagen Test Systems. Perhaps the most widely used single mutagen test system is that which uses specially constructed test strains of *Salmonella typhimurium*; this test is frequently referred to as the "Ames test." The details of this test have been extensively presented in the literature.[1,2,3] The popularity of this test among scientists, who are responsible for the evaluation of the potential mutagenicity of chemicals and drugs, lies in the fact that it is one of the least expensive mutagen tests and requires minimal amounts of space and equipment. Of course, a person well skilled in microbiological procedures is required to supervise the daily operations.

The Ames test utilizes several different histidine auxotrophic mutants

of *Salmonella typhimurium*. These bacteria are unable to carry out histidine biosynthesis and thus are dependent upon an external source of this amino acid for growth. In the presence of a mutagenic compound these test strains may revert back to the wild type, which are able to synthesize histidine. These strains have additional mutations in the cell wall to allow large molecules to permeate to the nucleus, thereby increasing their sensitivity to these kinds of chemicals. Seven test strains of *S. typhimurium* are currently being used and are identified as follows: strains TA1530 and TA1535 will detect base pair substitution mutations and strains TA1536, TA1537, and TA1538 will detect frameshift mutations. In addition, strains TA100, derived from TA1535, and strain TA98, derived from strain TA1538, have been further modified to enhance the sensitivity of these strains to base pair substitution mutations and frameshift mutations, respectively.[5]

In order to overcome a major shortcoming of the bacterial mutagen test, namely that mammalian metabolism is missing, most investigators incorporate a liver homogenate (termed "S-9") into the assay. Generally, this is prepared from rats induced with a polychlorinated biphenyl mixture (Aroclor 1254) or phenobarbital.[1] Other investigators, such as Brusick[6] have used homogenates prepared from other tissues and other species.

In addition to the test strains of *S. typhimurium*, other microbes systems such as *Escherichia coli*,[7] the fungus *Neurospora crassa*,[8] and the yeast *Saccharomyces cerevisiae*[9] have been used as indicator organisms in mutation tests. Two strains of *E. coli*, namely W3110 (polA$^+$) and the DNA polymerase mutant *E. coli* p3478 (polA$^-$) are used to detect genetic damage in bacteria by DNA repair. Cells which have reduced capability of repairing DNA may be more susceptible to the action of chemical mutagens as detected by increased heritable change. Such differential toxicity is taken as an indication that the chemical interacts with the DNA of the exposed cells to produce increased levels of genetic damage. *Saccharomyces cerevisiae* allows one to detect genetic damage in a eukaryotic microorganism whose chromosomes are structurally similar to the chromosomes of higher organisms but are too small to be observed directly.

Many workers feel that mutagenic tests with lower forms have little relevance to potential effects in man. To help bridge this gap, several workers are using urine and blood from treated animals or exposed humans in conjunction with the bacterial test systems.[10,11] The details of the procedures vary from investigator to investigator. Some use urine samples directly (after passing through a membrane filter in order to have an aseptic sample) on the plate. Others treat the urine with glucuronidase in order to split conjugated products, while other investigators might concentrate the metabolites through lyophilization or with the use of Amberlite columns. Similar treatments also may be done with serum. Nevertheless, even these modifications do not overcome many of the legitimate objections to these systems, *e.g.*, dosimetry, physiological barriers, *etc.*

Host-Mediated Assays. Although host-mediated assays were used rather extensively a few years ago to assess the potential mutagenic effects of chemicals, the assay has fallen into some dispute because of lack of sensi-

tivity because the pharmokinetics of the test agent in the host were not thoroughly understood.[98] Nevertheless, the assay does provide a definite bridge between microbial indicator organisms and a mammal. In the routine test the microbial indicator organism is injected into the peritoneal cavity of a mammal, usually a rodent. The host animal is then treated with the chemical by any route other than intraperitoneally. Several hours later the host is killed and the indicator organism is recovered and scored for mutants. Theoretically, a comparison can be made between the mutagenic action of the compound (1) on the organism directly, and (2) whether the host can detoxify the compound or whether mutagenic products (metabolites) are formed as a result of host metabolism. The histidine auxotrophs of *S. typhimurium* (the same test strains used in the Ames test) have been the most extensively used.

Gene Repair Systems. These systems are based on the hypothesis that DNA repair is an indirect measure of genetic damage.

A system which detects DNA damage in *E. coli* by repair of the genetic damage has been developed.[57] In addition to the use of bacteria, mammalian cells in culture have been employed. Stich[12] has described an assay in which cultured human skin fibroblasts are used to measure DNA repair by the unscheduled uptake of tritiated thymidine. The assay is based on the fact that DNA damage may be induced by chemical treatment of the cells and that this damage may be measured as an increase in DNA synthesis (DNA repair). Although Stich used human fibroblasts in culture, other investigators have used cell lines from Chinese hamsters, kangaroo rats, and Muntjac deer.

Sega[13] has described the details of a DNA repair system in early spermatid stages of the mouse. With four known mutagens, he has been able to detect DNA repair in early spermatids.

Dominant Lethal Test. Dominant lethal mutation is a genetic event that kills the individual which carries it. The damage, which notably consists of chromosomal-type mutations, is detected as preimplantation loss of non-viable blastocysts and as early embryonic death.[14] Fetal wastage above the spontaneous background rate is attributed to dominant lethal mutations since only the males are treated with the test compound; females can be treated also, although this test is more difficult to interpret. This test has been well evaluated by several investigators.[15-21]

Cytogenetic Assays. Cytogenetic assays refer to the general category of a wide variety of chromosome tests. Chromosomes are important in mutagenic studies since genes are physically located on the chromosomes. Several of the numerous cytogenetic assays available to the mutagenic investigator are directly applicable to man. That is, the same procedures that are used for treated animals can be applied to humans. Thus, these tests hold great promise for the evaluation of potential mutagenic compounds. Perhaps the most common cytogenetic assay involves short-term (48 or 72 hours) culture of lymphocytes.[22] Following well-established technique, geneticists have been able to further define inherited metabolic disorders in humans. Next to transmissible gene mutations, the chromosome mutations represent the

second major type of genetic change. This kind of genetic alteration is usually studied *in vivo* by using the short-term leukocyte cultures, or by using bone marrow cells directly. *In vitro* studies can be done with various cell lines such as those derived from Muntjac deer, Chinese hamsters, and mammalian tumor lines. Cytogenetic analyses allow the investigator to study the cells in metaphase or anaphase for structural changes and rearrangements of their chromosomes. The occurrence of such chromosomal aberrations might be correlated well with the administration of or exposure to known chemical mutagens and carcinogens. The details of several methods for these assays have been published.[23-27]

A recent addition to cytogenetic analyses involves the use of a staining technique which can detect sister chromatid exchanges.[28,29] This test gives a very sensitive and rapid method for the detection of chromosome damage by chemical agents and provides a powerful new method for detecting environmental mutagens. Sister chromatid exchange involves a symmetrical exchange at one locus between sister chromatids with no visible alteration of gross chromosome morphology.

Cytogenetic procedures have also been applied to study meiotic chromosomes.[30,31] Such procedures allow one to study chromosome changes within the spermatocyte by direct visualization of chromosome translocations.

In an effort to simplify cytogenetic assays, the micronucleus test was reported by Schmid.[32,33] This test is a relatively rapid *in vivo* method devised primarily for screening chemicals for chromosome breaking effects. All compounds which are clastogens ("chromosome breakers"), that have been studied, also cause increases in the numbers of bone marrow cells with micronuclei (small pieces of chromatin material).

Drosophila. The use of drosophila (fruit flies) for mutagen studies has several advantages. Few higher organisms can be reared in large numbers as easily and economically as can drosophila.[34,35] Thus, one can study many types of genetic alterations: dominant lethals, point mutations, chromosome rearrangements, and loss of X or Y chromosomes.

Specific Locus Test. The specific locus test in the mouse is a method for detecting and measuring rates of mutation at several recessive loci. The method has been described and reviewed extensively in the literature.[36-38] The method basically consists of mating treated and untreated wild type mice, either male or female, to a strain of mice homozygous for a number of known recessive genes. The recessive genes are such that they are readily expressed as visible phenotypes in the homozygous state. If a mutation has occurred in any of the test loci in the germ cells of the treated animals, it will be detected in the offspring. If no mutation has occurred following treatment, the progenies from the cross will be of the wild type.[39] The main disadvantage of this test is that it requires the production of large numbers of mice and consequently considerable space for housing.

Translocation Test. Heritable translocations can be measured in animals. For convenience, male mice are usually used. The assay consists of deter-

mining the ability of a chemical to induce reciprocal translocations in the germ cell lines of treated mice.[16,40] Heritable translocations can be observed in dividing spermatocytes of F_1 males or in the offspring of F_1 females.

The Tier Approach. The tier approach has been suggested as strategy for setting priority when large numbers of chemicals must be evaluated, or if one is choosing between analogs for further development.[41,42] These hierarchal schemes expedite the testing and evaluation of genetically hazardous substances in a stepwise fashion beginning with the most rapid and inexpensive assays continuing through the more time-consuming and costlier tests until a definitive assessment appropriate to the compound in question is determined. The first tier contains short-term screening tests with submammalian systems, the second tier contains short and longer term tests with whole animals, and the third tier involves a risk-benefit evaluation which may entail further, more specialized testing procedures and experiments on the detailed metabolism of the agent *in vivo*. In the pharmaceutical industry where a manageable number of substances are evaluated for potential use, several tests from the various tiers may be applied, almost simultaneously. Regardless of which studies are used, the results of all studies must be put into proper perspective prior to the widespread use of those substances which have, in fact, given positive responses in certain mutagenic tests.

It is apparent that chemicals which interact with DNA or are metabolically converted to substances which have the capacity to interact with DNA through electrophilic attack or by other means to alter DNA have the potential to induce mutation. It is also apparent from knowledge of chemistry and biochemistry that many substances, man-made and natural, will have this capability either directly or following metabolism of the chemical by the host organism.

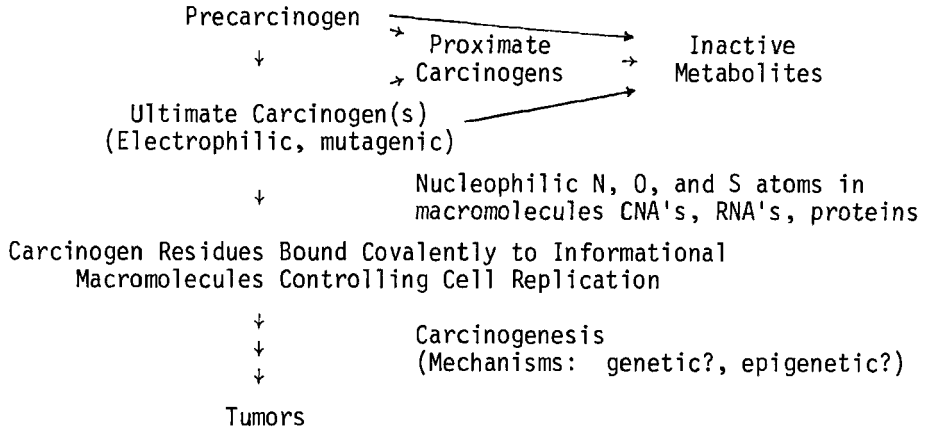
The use of mutagenic test systems to identify possible chemical carcinogens is based on three major developments in the fields of chemical carcinogenesis and chemical mutagenesis: (1) the recognition that chemical carcinogens may be divided into two broad categories--precarcinogens and ultimate carcinogens; (2) the successful coupling of metabolic activation and gene mutations *in vitro*; and (3) a background of information in the literature indicating that a high number of chemical carcinogens with greatly varied structures were mutagenic.

The somatic mutation concept requires that carcinogens are also mutagens and that carcinogenic and mutagenic potentials of carcinogens should be correlatable. These problems were reviewed by Burdette[43] in 1955, at which time he concluded that there was no correlation between the mutagenic and carcinogenic activities of those chemicals which had been assayed for both types of activity. In 1955, very little was known with regard to structures of the ultimate reactive forms of chemical carcinogens *in vivo*, but Burdette recognized that the lack of correlation could be due, among other factors, to differences in the metabolic fates of the chemicals in the different organisms used to assay carcinogenicity and mutagenicity.

In 1966, the state of knowledge and confusion of the relation between mutagenesis and carcinogenesis by chemicals had not changed much from that described by Burdette[43] and led the Millers[44] to conclude that there is no clearly established relationship between the mutagenic and carcinogenic activities of chemicals. Certain alkylating agents were shown to possess both properties, but several strong mutagens and strong carcinogens had been tested for carcinogenicity and mutagenicity, respectively, with negative results.

In recent years it has become evident that the majority of chemical carcinogens are precarcinogens which must be converted *in vivo* to ultimate carcinogens. These metabolic conversions are usually mediated by oxidative enzymes and may involve the formation of intermediate metabolites termed proximate carcinogens. Although chemical carcinogens comprise a wide variety of structures and for a long time it was difficult to visualize a common denominator for carcinogenic activity, it now appears that

Fig. 1. SCHEME FOR THE METABOLIC ACTIVATION AND DEACTIVATION OF CHEMICAL CARCINOGENS AND FOR THE ROLE OF ULTIMATE CARCINOGENS[45]

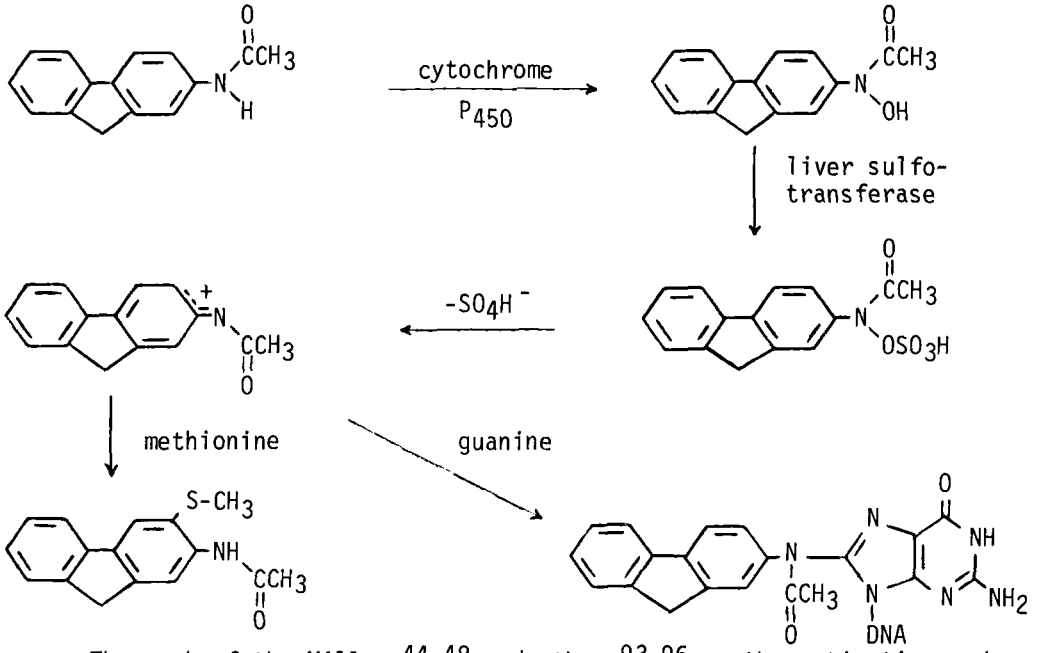


the ultimate carcinogenic forms have the common feature that most, if not all, are strong electrophilic reactants.[46,47] That is, the ultimate carcinogens appear to contain relatively electron-deficient atoms which can react covalently with electron-rich or nucleophilic atoms in cellular components including those in macromolecules such as the nucleic acids (DNA and RNA) and proteins. The apparent identity of the ultimate electrophilic reactants and the ultimate carcinogenic forms of chemical carcinogens was first evident for the simple carcinogenic alkylating agents. The similarities in their reactions to those of the alkylating agents strongly implicated alkylating intermediates as the ultimate carcinogenic forms of the alkylnitrosamines, alkylnitrosamides, dialkylhydrazines, dialkyltriazines, and cycasin.[45]

The electrophilic concept of chemical carcinogenesis was extended

primarily from investigations on the metabolism of 2-acetylaminofluorene (AAF) and 4-methylaminoazobenzene (MAB). The carcinogenicity of the aromatic amines and amides that have been adequately studied depends on their conversion to N-hydroxy derivatives and then on the conversion of the latter metabolites to electrophilic metabolites.[46,48] The metabolic activation of the liver carcinogen AAF and its proximate metabolite N-hydroxy-AAF has been studied in considerable detail. Esterification of N-hydroxy-AAF by a 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-dependent system in the soluble fraction of rat liver converts N-hydroxy-AAF to a very reactive and mutagenic sulfuric acid ester, which also appears to be the major ultimate carcinogenic metabolite for the liver.[49-51] Other electrophilic metabolites of N-hydroxy-AAF (glucuronide, acetate, and phosphate) are also formed enzymatically by various tissue preparations and should be considered as possible ultimate carcinogens in some situations.[48,52-54]

Fig. 2. METABOLIC ACTIVATION OF 2-ACETYLAMINOFLUORENE (2-AAF) AND REACTIONS OF THE ELECTROPHILIC INTERMEDIATE WITH CELLULAR CONSTITUENTS[50]



The work of the Millers[44-48] and others[93-96] on the activation and reactivity of chemical carcinogens led to the recognition and acceptance of the fact that, with the exception of direct alkylating agents, chemicals are not carcinogenic *per se* but must undergo metabolic activation by mammalian enzymes. This knowledge contributed greatly to the development of the current *in vitro* assays since it revealed that the failure to detect mutagenic activity in many of the early genetic tests could be directly attributed to the fact that bacteria do not duplicate mammalian metabolism in activating carcinogens since they lack many of the appropriate enzymes

required to activate the chemical to its biologically active forms. Consequently, the failure to detect any correlation between carcinogenesis in animals and mutagenesis in bacteria from the early studies is understandable.

The first published work in which the mutagenic activity of metabolites was detected after metabolic activation of carcinogens was by Malling[55] who used mammalian-liver homogenates to activate dimethylnitrosamine to a compound that reverted *Salmonella typhimurium* test strains. Shortly thereafter, mammalian-liver homogenates were used by Gainer[56] to activate aflatoxin B, to a compound lethal to a *Salmonella* test strain lacking excision repair, and by Slater[57] to activate dimethylnitrosamine to a compound lethal for *E. coli* bacteria lacking polymerase I. Subsequently, Ames[3] extended this earlier work by adding human, or rat liver homogenates and a TPNH-generating system directly to petri plates with his *Salmonella typhimurium* test strains and the carcinogen. These procedures have been further modified to include liver homogenates obtained from animals whose liver enzymes have been induced with phenobarbital, Aroclor, or 3-methylcholanthrene in order to provide for efficient detection of a variety of carcinogens requiring metabolic activation.

As a result of the advances made in the development of the microbial systems for detecting mutagens, a high correlation between mutagenicity in these assays and carcinogenicity in animals[3,55,57,64,71] became evident. The fact that chemical carcinogens are also mutagens should not be particularly surprising since apparently both biological end points are reached through an electrophilic attack on DNA by the parent compound, in the case of a direct acting carcinogen, or a metabolite form of the chemical. The concept of the reactive electrophilic agent forms the rationale behind the utilization of mutagenicity assays in screening for potential carcinogens. If chemical carcinogens or their electrophilic metabolites induce genetic changes which directly or in association with other cellular dysfunctions result in the malignant transformation of normal cells to potential tumor cells, then by the detection of mutagenic activity potential carcinogens could be identified.

In 1975 Ames published the results of his study of 300 compounds in the *Salmonella*/microsome mutagenicity test in which he demonstrated that 90% (156/174) of the carcinogens were also mutagenic in the assay. Despite the severe limitations inherent in defining non-carcinogenicity of compounds, few non-carcinogens (<10%) showed any degree of mutagenicity.[58-60]

The Ames test has demonstrated the ability to detect a variety of carcinogens as mutagens, such as direct alkylating agents, nitrosamines, polycyclic hydrocarbons, fungal toxins, aromatic amines, nitrofuran carcinogens, a variety of antineoplastic agents, and antibiotic carcinogens such as adriamycin, daunomycin, and mitomycin C. In addition, most of the known human chemical carcinogens which have been tested were positive. These include β-naphthylamine, benzidine, cigarette smoke condensates, bischloromethyl ether, aflatoxin B_1, vinyl chloride and 4-aminobiphenyl.[58]

The present data indicate that approximately ten percent of the car-

cinogens (17/175) were non-mutagenic in the test (Table 1).

TABLE 1. NON-MUTAGENIC CARCINOGENS[59]

o-toluidine	ethyl carbamate	safrole
auramine	3-amino-1,2,4-triazole	cycasin
carbon tetrachloride	phenobarbitol	4-aminoantipyrine
DDE	thioacetamide	1,2-dimethylhydrazine
dieldrin	acetamide	procarbazine
thiourea	ethionine	

Some modifications in the *in vitro* metabolic activation system may be necessary for the detection of some of these carcinogens as mutagens, for example, the chlorinated hydrocarbons (carbon tetrachloride, DDE, and dieldrin), ethyl carbamate, safrole, procarbazine and 1,2-dimethylhydrazine, while in the case of auramine, 4-amino-antipyrine and acetamide, the carcinogenicity studies in animals have not been definitive.[59] 3-Amino-1,2,4-triazole, thioacetamide and thiourea possess goitrogenic activity and Weissburger has suggested that these agents cause thyroid tumors through a non-mutagenic mechanism.[61] Cycasin, a β-glucoside of methylazoxymethanol, is inactive in the Ames procedure because neither *Salmonella* nor mammalian microsomes contain a β-glucosidase necessary for converting it to methylazoxymethanol which does show mutagenic activity in the assay; while ethionine mutagenicity may not be detectable in *Salmonella* since it may act as a carcinogen by ethylating nucleic acid at natural methylation sites through S-adenosylethionine.[59]

The Ames test appears to be highly selective for the detection of carcinogens. To date, approximately 108 noncarcinogens (chemicals negative in tests for carcinogenicity in animals), falling into two general categories--62 chemicals, most of which are closely structurally related and even isomeric to carcinogens, and 46 common biochemicals--have been tested.[58-60] About 13% of these noncarcinogens showed some degree of mutagenic activity in the assay; however, since carcinogenicity studies have been extremely limited for several of these compounds, there must be doubt as to the classification of these chemicals as noncarcinogens.

Other microbial mutation, repair and recombination assays have also been used in chemical screening, however, the data base for carcinogenicity-mutagenicity correlation established with these *in vitro* test systems is relatively limited at this time.[62-70] At present, the Ames *Salmonella* assay appears to be the most generally applicable screen for the detection of chemical mutagens. No other mutagenesis assay has been shown to respond to such a wide group of chemical types as the *Salmonella* mutants.

The *in vitro* mutagenicity assays with Ames *Salmonella typhimurium* strains have been used to investigate the extent and underlying basis for organ, species, and sex variability in carcinogen metabolism and sensitivity to the action of chemical carcinogens. Recently Weekes and Brusick[71] used the *in vitro* system to compare the relationship between target organ susceptibility for dimethylnitrosamine-induced tumors and *in vitro* metabolic acti-

vation of dimethylnitrosamine (DMNA) to a mutagen. DMNA has been shown to be both mutagenic and carcinogenic following metabolic activation *in vivo* and mutagenic following activation *in vitro*.[72,73] In addition, Czygan[74] demonstrated that the rate of mutagen formation was linked to the rate of enzymatic conversion of DMNA thereby permitting the mutation assay to be used as an indirect assay for the enzymatic activity of a particular tissue to biotransform DMNA into a mutagenic (carcinogenic) intermediate. In mice, DMNA induces predominantly liver and lung tumors with low frequencies of kidney tumors and rarely, if ever, tumors of the spleen or gonads.[72,75-77] The data generated by Weekes and Brusick[71] demonstrated that activation of DMNA to a mutagenic intermediate by microsome preparations from these organs of BALB/cJ, RF/J and C57Bl/6J mice show the same ranking (liver > lung > kidney > spleen > testes) suggesting that at least for the carcinogen DMNA, the level of metabolic activations of a tissue may be directly related to the susceptibility of the organ as a target for tumor induction.

Species differences in drug metabolism have also been linked to observed variability in target organ sensitivity to the tumorigenic action of DMNA. In mice, C3H and Swiss strains are highly susceptible, showing renal tumor incidences of approximately 16 and 11 percent respectively, while BALB/c and RF strains are quite resistant showing renal tumor frequencies lower than 4 percent.[75-77] Recently, Weekes[78] was able to show that when microcome preparations from the kidneys of these four strains were compared for their ability to activate DMNA to a mutagen, there was an excellent correlation between carcinogenic susceptibility and mutagen production.

Kidney microsomes from the two mouse strains exhibiting high susceptibility to DMNA-induced renal tumors were considerably more active in forming the mutagenic/carcinogenic intermediate than preparations from the two strains with lower sensitivity to DMNA. In a similar fashion, Weekes and Brusick[71] were able to show that the mutagen data from microsome preparations from liver and lung tissues from all four strains were similar as would be expected since the liver and lung tumor susceptibilities of these mouse strains to DMNA are nearly equivalent. These data tend to confirm the initial assumption that the four mouse strains exhibit different levels of DMNA activation and that target organism metabolism of DMNA is important for the initiation of neoplasia.[71,78]

In a comparison of the metabolic activation of dimethylnitrosamine by liver, lung, and kidney microsomes from male and female C57BL/6J mice, the existence of a sex difference in DMNA activation with kidney microsomes was demonstrated in that the female mice could not metabolize DMNA to a mutagen.[71] Similar sex differences were not observed in studies with Sprague-Dawley or Fischer rats.

Hormonal regulation of mouse kidney activations was subsequently demonstrated by Brusick.[6] Neither male nor female kidney preparations were able to activate DMNA to a mutagen until they were 35 days old, at which time the activation potential in male kidney preparations began to increase. It was concluded that male hormones might be responsible for the regulation since the activation timing (day 35) closely coincides with the onset of testosterone synthesis and the expression of adult behavior in

male mice. In studies with TFM (testicular feminization males), the affected male animals who appear phenotypically as females were similar to female animals in their metabolic activation of DMNA thereby substantiating the hormonal regulation of mouse kidney activations.

Species, sex, and target-organ differences were also noted in the activation of aromatic amines. These compounds were found to be activated to mutagens by mouse kidney microsomes to a greater extent than by mouse liver. With the carcinogen 2-acetylaminofluorene (AAF), Brusick[79] reported that male mice of several strains showed approximately 10 times greater activation of AAF to a mutagen by kidney microsome preparations than by liver preparations. This difference was not shown for other species such as the rat, guinea pig, dog, rabbit, or monkey. Miller[80] has reported that AAF induces a high incidence of bladder tumors in mice and this observation may be consistent with their high rate of kidney activation for AAF. The mouse kidney activation of AAF has also been shown to be under hormonal regulation in that the activation potential of the TFM males was lower than the normal male activation potential and almost exactly as low as female mouse kidneys.[79] Additional studies demonstrating differences in target-organ metabolic activation of nitrosamine and chlorinated hydrocarbon carcinogens have recently been reported by Bartsch.[81]

The use of *in vitro* microbial mutagenesis assays to follow metabolic transformation of chemical carcinogens (precarcinogens) has generated data that augments the reliability of the empirical correlation between carcinogenesis and mutagenesis originally demonstrated in the reports by Ames.[1,58] In addition, the results of Brusick[79] show a quantitative relationship between microsomal enzyme activation and tumor susceptibility for DMNA and AAF in mice strongly implicating the active intermediate as both the ultimate carcinogen and mutagen. The results of these studies provide additional support to the theory that a chemical-DNA interaction is a prerequisite for at least some tumor inductions and that there is a functional link between mutagenic and carcinogenic events.

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Chapter 25. Brain Neurotransmitter Receptor Binding and Neuroleptic Drugs

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The molecular mechanism of action responsible for the efficacy of a therapeutic drug may be suggested by the biochemical event which correlates best with clinical potency. The past year has seen the successful characterization of the CNS dopaminergic and noradrenergic α-receptors by means of binding studies utilizing radioactively labeled receptor-specific ligands. The interactions of neuroleptic drugs with these binding sites have provided insight into the way that neuroleptics produce their unwanted side effects as well as their therapeutic mode of action. Such binding studies may provide excellent *in vitro* screens predictive of antischizophrenic potency, the degree of extrapyramidal and autonomic sympatholytic side effects and the likelihood of tardive dyskinesia following chronic treatment.

Neuroleptic Interactions with the Dopamine Receptor - Binding studies, by definition, label the recognition site of a receptor. The striatal dopamine-sensitive adenylate cyclase, which encompasses both a recognition and an effector unit, initially held promise as a biochemical marker of the dopamine receptor which might provide a unitary explanatory principle for the therapeutic mode of action of neuroleptic drugs[1,2]. However, the lack of correlation between clinical potency and inhibition of dopamine stimulated cAMP production across all groups of neuroleptic drugs believed to act by blocking dopamine receptors[3,4], has indicated that the interactions between the recognition site and the adenylate cyclase must be complex[5].

Two studies attempted to label the recognition site of the dopamine receptor with radioactively labeled neuroleptic drugs but were unsuccessful[6,7]. This was, in part, because most radioactive ligands can bind to biological membranes in relatively nonspecific manners such as by hydrophobic interactions, ionic attraction or van der Waal's forces. Since the number of such nonspecific binding sites is virtually infinite it is difficult to distinguish specific receptor binding (in the order of 10-100 pmole/g wet wt tissue) from the nonspecific background. To overcome this problem it is necessary to use low concentrations of a high specific activity labeled ligand which has high receptor affinity so that the ratio of specific to nonspecific binding can be maximized. It is necessary to rinse tissue preparations after incubation by rapid filtration on glass fiber filters under vacuum to remove the lower affinity, nonspecifically bound ligand[8].

Recently in ours and Seeman's laboratory the binding of ^{3}H-haloperidol to the dopamine receptor has been characterized[9,10]. In general a crude membrane preparation from calf striatum has been used. Binding occurs rapidly at 37° C and reaches equilibrium between 1 and 3 min with half maximal binding attained at about 20 sec. There are three major criteria which must be satisfied in order to demonstrate that the haloperidol

binding sites are in fact the physiologically and pharmacologically relevant dopamine receptor. These are 1) Saturability: ligand binding must be saturable indicating a finite number of binding sites. ^{3}H-Haloperidol binding displaceable by high concentrations of dopamine has a K_D of 1-3 nM determined in both equilibrium and kinetic studies saturating between 10-15 nM[11]. However the displacement of ^{3}H-haloperidol with nonradioactive haloperidol indicates multiple components of binding. Between 30-60% of total striatal binding (depending on species) occurs to the specific dopamine receptor, while the remainder occurs to nonspecific sites including other neurotransmitter receptors and uptake sites for which haloperidol has been shown to have low affinity. The specific binding to the dopamine receptor can be distinguished from such nonspecific binding by pharmacological techniques (see below). 2) Regional localization: binding sites are expected to be found only in areas where the neurotransmitter is present. However, this does not necessarily imply a perfect correlation between the level of a neurotransmitter and the concentration of its receptors in various brain areas. ^{3}H-Haloperidol binding is highest in areas which are known to have a large dopamine innervation such as the caudate nucleus. Lower numbers of binding sites occur in the globus pallidus, putamen, olfactory tubercle and nucleus accumbens[11]. ^{3}H-Haloperidol binding has also been detected in the cerebral cortex[12] but not in the thalamus, hippocampus or cerebellum where there is no known dopamine innervation. Seeman has recently detected binding in the pituitary where the dopamine receptor sites may be involved in the control of prolactin release[13]. 3) Pharmacological specificity: dopamine agonists and antagonists which differ in potency in in vivo, behavioral and pharmacological tests should exhibit parallel differences in potency in competing for ^{3}H-haloperidol binding sites. This criterion takes on added importance in the case of ^{3}H-haloperidol binding which occurs to multiple sites. Optical isomers of neuroleptic drugs which have markedly different clinical potencies also exhibit isomeric specificity in their competition for ^{3}H-haloperidol binding and can be used to define stereospecific receptor binding to the pharmacologically relevant dopamine receptor. One such drug is butaclamol, a new antischizophrenic agent which exists as optical isomers, with virtually all the dopamine blocking activity residing in the (+)-isomer[14]. The maximum stereospecific difference between the binding of ^{3}H-haloperidol in the presence of (+)-butaclamol and that in the presence of an equal concentration of (-)-butaclamol is thus a measure of the stereospecific binding of ^{3}H-haloperidol to the dopamine receptor. (+)-Butaclamol displays two clearly distinct components in inhibiting ^{3}H-haloperidol binding. The high affinity component elicits half maximal effects at about 1 nM while the lower affinity component is only apparent at concentrations above 1 μM. By contrast, (-)-butaclamol lacks the high affinity component of inhibition of ^{3}H-haloperidol binding while its lower affinity inhibition of ^{3}H-haloperidol binding resembles the low affinity influence of (+)-butaclamol[10,11]. Dopamine maximally reduces haloperidol binding to the same extent as the high affinity component of (+)-butaclamol inhibition. The inhibitions by these maximally inhibiting concentrations of butaclamol and dopamine are not additive indicating that both drugs are competing for the same class of ^{3}H-haloperidol binding sites[11]. The agonist specificity of ^{3}H-haloperidol binding is consistent with dopamine receptor pharmacology: apomorphine and

dopamine being more potent than (-)-epinephrine and (-)-norepinephrine while isoproterenol is essentially inactive[9,10,11].

Dopamine Receptor Binding Predicts Clinical and Pharmacological Potencies of Neuroleptic Drugs - An abundance of recent research suggests that neuroleptic drugs may exert their therapeutic actions and induce extrapyramidal side effects by blocking dopamine receptors in the brain[2,15]. While molecular modeling indicates how phenothiazines can assume the preferred conformation of dopamine[16], the conformation of butyrophenones at their receptor sites is unclear. Nevertheless since both phenothiazines and butyrophenones have many behavioral and biochemical effects in common it has been assumed that they exert their therapeutic effects by a similar mechanism. Spiroperidol is the most potent inhibitor of ^{3}H-haloperidol binding that we have studied with a value of the inhibition constant, K_i indicating 50% receptor occupation, of 0.25 nM. It has a 5-fold higher affinity for ^{3}H-haloperidol binding sites than fluphenazine, a potent phenothiazine, a 40-fold greater affinity than chlorpromazine and a 125-fold to 950-fold greater affinity than the weak neuroleptics pipamperone, promazine and promethazine[11].

In examining a large number of phenothaizines, butyrophenones and other neuroleptic agents both we and Seeman have demonstrated that there is an excellent correlation between the molar pharmacological potencies of these agents in animals and man and their affinities for ^{3}H-haloperidol binding sites[17,18,19]. The affinities of the drugs that we have examined for ^{3}H-haloperidol binding sites correlate highly with their molar potency in antagonism of both apomorphine-induced stereotypy (r= 0.94, $p < 0.001$) and amphetamine induced stereotypy in the rat ($r=0.92$, $p < 0.001$)[17]. Similarly blockade of apomorphine-induced emesis in dogs, which is one of the most sensitive *in vivo* neuroleptic assays and is presumed to involve stimulation of dopamine receptors in the vicinity of the chemoreceptor trigger zone in the brainstem, correlates closely with ^{3}H-haloperidol binding site affinity ($r=0.93$, $p < 0.001$) all animal *in vivo* data from Janssen[20]. It is dramatic that clinical potency as antipsychotic agents in man simply determined by the average daily oral

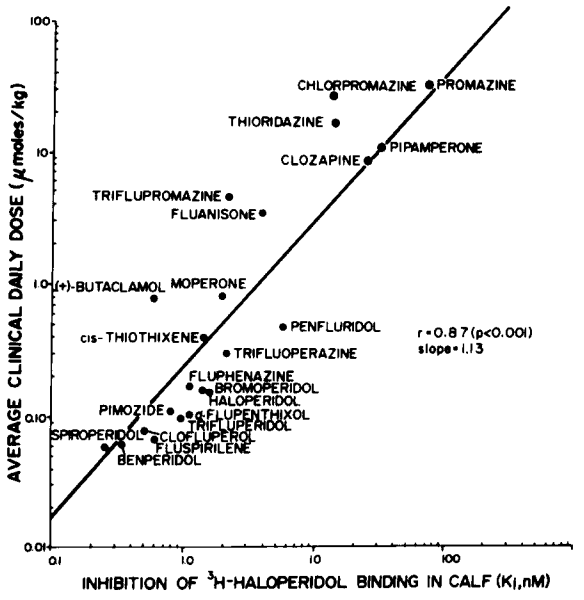


Fig. 1 Correlation between antischizophrenic potency and ^{3}H-haloperidol binding (reprinted from Ref. 18. Copyright 1977 by the A.A.A.S.)

effective dose, correlates very highly with competition for ^{3}H-haloperidol binding ($r=0.87$, $p < 0.001$)[17,18,19] (Fig. 1). These impressive correlations indicate that the affinity of a drug for the ^{3}H-haloperidol binding site of dopamine receptors is a powerful predictor of clinical activity. The correlations are all the more impressive because binding studies were conducted *in vitro* and animal behavioral and human studies conducted *in vivo*. Seeman has calculated that these inhibition constants determined *in vitro* are very similar to the plasma concentrations of the antipsychotic drugs at therapeutic dose levels[19] further reinforcing the concept that the blocking of dopamine receptors is responsible for their antipsychotic activity.

Extrapyramidal Side Effects and Muscarinic Binding - It has been hypothesized that dopamine receptor blockade in the corpus striatum is responsible for the extrapyramidal side effects of neuroleptic therapy while dopamine receptor blockade in the limbic forebrain, regions classically associated with emotional behaviors, is responsible for the antischizophrenic efficacy of neuroleptic agents[15]. However, regional studies of ^{3}H-haloperidol binding have not indicated any fundamental differences in dopamine receptor binding between the corpus striatum, olfactory tubercle or nucleus accumbens[17]. Some neuroleptics, such as thioridazine and clozapine, elicit a much lower incidence of extrapyramidal side effects than most commonly used neuroleptics. It is unlikely that this results from differential influences on dopamine receptors in various areas because regional studies indicate that clozapine and thioridazine have about the same affinity for ^{3}H-haloperidol binding sites in the corpus striatum and in the limbic dopamine areas. The relative affinities of clozapine and thioridazine for ^{3}H-haloperidol binding in relation to other neuroleptics corresponds reasonably well with their clinical potency indicating that these drugs probably exert their antischizophrenic effects by a similar mechanism to the other antipsychotic agents. Thus one would expect that when given in therapeutic, antischizophrenic doses these drugs should all produce the same incidence of extrapyramidal side effects.

Recent studies of the muscarinic acetylcholine receptor in the brain may provide a resolution of this dilemma[21,22]. It is well-known that concurrent administration of anticholinergic drugs is especially effective in antagonizing the extrapyramidal side effects of neuroleptics without apparently reducing their antipsychotic potency[23]. The therapeutic efficacy of the anticholinergics apparently reflects a balance in the corpus striatum between dopamine and acetylcholine involved in motor control such that antagonizing acetylcholine effects is equivalent to enhancing those of dopamine, and vice versa. Thus if neuroleptics varied in their anticholinergic properties they may well vary in their propensities to induce extrapyramidal side effects. In studies of the binding of 3-quinuclidinyl benzilate (QNB), a potent antagonist of muscarinic cholinergic receptors, to striatal membrane preparations, this hypothesis was confirmed. Clozapine which is almost devoid of extrapyramidal side effects has the greatest affinity for muscarinic receptors, similar to that of classical anticholinergic agents. Thioridazine, which next to clozapine elicits the fewest extrapyramidal symptoms is second most potent. The alkylamino phenothiazines, whose moderate incidence of extrapyramidal actions is greater than

that of thioridazine, have correspondingly less affinity for the acetylcholine receptor. Piperazine phenothiazines and the butyrophenones, whose frequency of extrapyramidal effects is greatest have the least affinity for the muscarinic receptor[21]. According to this hypothesis, when given at therapeutic antischizophrenic doses all neuroleptics produce comparable dopamine receptor blockade and thus all do have about the same tendency to elicit extrapyramidal side effects. The simultaneous blockade of acetylcholine receptors by drugs such as clozapine and thioridazine antagonizes these extrapyramidal side effects while, because of their negligible anticholinergic activity at normal doses,drugs such as haloperidol elicit many more extrapyramidal side effects. This phenomemon may also explain a paradoxical response seen in some schizophrenic patients who are refractory to normal doses of drugs such as haloperidol. As the dose is increased marked extrapyramidal side effects occur only to disappear as the drug dosage reaches abnormally high levels. It is possible that at such high dose levels the drug's weak intrinsic anticholinergic activity is now sufficient to antagonize the extrapyramidal side effects.

Screening of potentially useful antipsychotic drugs for muscarinic receptor affinity may thus provide a simple in vitro predictor of their propensity to induce extrapyramidal side effects.

Tardive Dyskinesia and Dopamine Receptor Supersensitivity - Tardive dyskinesia is a major complication of long term treatment with neuroleptic drugs [24,25,26,27]. It is characterized by abnormal movement of facial muscles and extremities which frequently worsen when the neuroleptic dose is lowered or terminated. Increasing the dose, however, may temporarily alleviate the symptoms. The chronic treatment of rats and mice with neuroleptics leads to an increased motor activity and enhanced sensitivity to the motor stimulant effects of apomorphine, a direct dopamine receptor agonist[28,29,30,31,32]. A similar motor supersensitivity to dopamine receptor stimulants is apparent when dopamine synaptic activity is reduced by inhibiting synthesis of dopamine with α-methyltyrosine[33] or by depleting dopamine storage sites with reserpine[34]. Since lesioning the nigrostriatal dopamine pathway also produces an enhanced sensitivity to dopamine receptor agonists which is hypothesized to result from a dopamine receptor supersensitivity[35], speculations have linked the development of tardive dyskinesia with the supersensitivity of dopamine receptors after prolonged blockade by chronic drug administration[25,36].

In order to investigate this hypothesis directly, rats were treated for 3 weeks with the potent butyrophenone neuroleptic haloperidol (0.5 mg/kg/day). Five days after terminating the chronic treatment there was a highly significant ($p < 0.001$) 20% increase in specific haloperidol binding [37]. Fluphenazine, one of the most potent phenothiazine neuroleptics, produced a similar increase in binding after administration at the same dose level for 3 weeks. By contrast, treatment for 3 weeks with a 5-fold higher dose of the phenothiazine promethazine, which lacks antischizophrenic activity, failed to significantly enhance ^{3}H-haloperidol binding. Depletion of brain dopamine by chronic administration of reserpine (0.25 mg/kg/day) also produced a similar 20% augmentation of ^{3}H-haloperidol binding.

The increase in binding seen after one week of haloperidol treatment was similar to that after 3 weeks. Twelve days after terminating haloperidol treatment the increased binding was less apparent than 5 days after termination, while at 17 days no increase was detected.

The enhanced receptor binding could be a reflection of either an increased number of binding sites or change in their affinity. Scatchard analysis of saturation data from striatal samples of individual rats indicate that 5 days after terminating the administration of haloperidol (0.5 mg/kg/daily for 21 days) the dissociation constant for ^{3}H-haloperidol binding showed no significant change. By contrast, there was a 20-25% increase in the total number of binding sites which is statistically significant ($p < .005$).

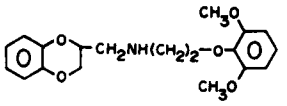
These data indicate that the motor changes seen after chronic neuroleptic treatment are associated with an increase in the number of dopamine receptor sites. This increase in the number of ^{3}H-haloperidol binding sites is consistent with the behavioral supersensitivity to apomorphine in rats treated with a similar dose schedule of neuroleptics. However the greater relative enhancement of apomorphine stimulant effects in such rats compared to the increased dopamine receptor binding described here indicates that other components in the overall system determining the behavioral response may also be changed during chronic neuroleptic treatment producing additive effects. However, the activity of the dopamine-sensitive adenylate cyclase in the corpus striatum is not altered in mice treated chronically with neuroleptics[32]. Moreover, the ability of apomorphine to elevate striatal cyclic AMP concentrations *in vivo* is unaltered in these mice[32].

Behavioral supersensitivity to apomorphine is more pronounced after lesions of the nigrostriatal dopamine pathway than after chronic treatment with neuroleptic drugs. Consonant with this, we have found that following a unilateral 6-hydroxydopamine-induced lesion of the dopamine cell bodies in the substantia nigra there is a 20-120% increase in striatal ^{3}H-haloperidol binding 2-7 mos. following the lesion[38]. This increased ^{3}H-haloperidol binding is also the result of an increased number of binding sites. Rats which are more behaviorally supersensitive also tend to show a greater increase in dopamine receptor binding.

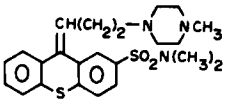
It is possible that enhanced dopamine receptor binding following chronic drug treatment may be a useful *in vitro* predictor of *in vivo* propensity to produce tardive dyskinesia.

Neuroleptic Interactions with the Noradrenergic α-Receptor - Autonomic sympatholytic effects such as orthostatic hypotension and sedation are among the most prominent untoward actions of neuroleptic drugs. These side effects have been attributed to blockade of central and peripheral adrenergic α-receptors[39,40,41] but direct quantitative evaluation of α-receptor blockade in the CNS by these agents has not been heretofore feasible. In the past year it has become possible to label the α-receptor for norepinephrine with a specific antagonist ^{3}H-WB-4101[42,43]. Binding is saturable

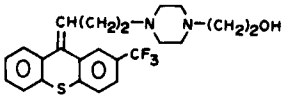
with a K_D of around 0.5 nM. Agonist potency in inhibiting ^{3}H-WB-4101 binding corresponds to the relative activity of these agents at α-receptors in the periphery: epinephrine > norepinephrine > > isoproterenol. Competition for binding is stereospecific with (-)-isomers of phenylethanolamines several times more potent than the corresponding (+)-isomers. Classical α-antagonists inhibit binding of ^{3}H-WB-4101 at low concentrations in the nM range while β-antagonists are much weaker. There is little regional variation in the amounts of ^{3}H-WB-4101 binding although levels are highest in the cortex and about 40% lower in the cerebellum[43].



WB-4101



THIOTHIXENE



FLUPENTHIXOL

Some neuroleptics are highly potent in competing for ^{3}H-WB-4101 binding to α-receptor sites in the rat brain[44]. Recent studies of a norepinephrine-sensitive adenylate cyclase in rat limbic forebrain have also indicated that the neuroleptics have a high affinity for norepinephrine α-receptor sites[45,46]. The most potent drug is the butyrophenone droperidol whose K_i value for inhibiting ^{3}H-WB-4101 binding is 0.7 nM. All the commonly used neuroleptics display K_i values under 50 nM putting them in the same range of potency as the classical α-adrenergic antagonists phentolamine and phenoxybenzamine, whose K_i values are 3.6 and 4.0 nM respectively [44]. Thus the neuroleptics, as a general class, are approximately equipotent in displacing labeled α-receptor and dopaminergic receptor antagonists. This similarity is further underlined by the finding that neuroleptics also manifest stereoselectivity in competing for ^{3}H-WB-4101 binding. Among neuroleptics with geometric isomers, neuroleptic action in intact animals is manifested selectively by cis-thiothixene and α-flupenthixol but not by their trans- or β-isomers. Cis-thiothixene is about 100 times more potent than trans-thiothixene and α-flupenthixol is about 50 times more potent than β-flupenthixol in inhibiting ^{3}H-haloperidol binding. These isomeric differences are maintained, although to a substantially lesser extent, in inhibiting ^{3}H-WB-4101 binding where the active isomers of these drugs are 23-fold and 3.6-fold more potent respectively. (+)-Butaclamol has an affinity of 35 nM for ^{3}H-WB-4101 binding and is 70 times more potent than its (-)-isomer[47]. (+)-Butaclamol exhibits a greater than 1000-fold stereoselectivity at ^{3}H-haloperidol binding sites[11].

These results emphasize that one must critically examine how reliably stereospecificity and correlations between clinical and biochemical effects can reveal the biochemical basis of neuroleptic drug action. We investigated the stereospecific influences on receptor binding for ^{3}H-GABA, for the opiates ^{3}H-naloxone and ^{3}H-dihydromorphine, for binding of ^{3}H-strychnine to the glycine receptor, for the binding of ^{3}H-QNB to the muscarinic cholinergic receptor, for ^{3}H-dihydroalprenolol binding to the noradrenergic β-receptor and for ^{3}H-serotonin and ^{3}H-lysergic acid diethylamide (LSD) to

the serotonin receptor[48]. Only the binding of ^{3}H-LSD and ^{3}H-serotonin is also inhibited stereospecifically and with considerable potency by these neuroleptics. Thus (+)-butaclamol is 140 times as potent (IC_{50} 50 nM) as (-)-butaclamol in lowering ^{3}H-LSD binding. This neuroleptic is somewhat less potent in lowering the binding of ^{3}H-5-HT (1 μM) but still displays a degree of stereospecificity. However since the neuroleptics are at least 50 times weaker at inhibiting ^{3}H-LSD binding as they are in inhibiting ^{3}H-haloperidol binding, in spite of these stereospecific effects, blockade of 5-HT receptors is probably not a major source of neuroleptic clinical efficacy. The relative influences of an extensive series of these drugs on ^{3}H-LSD binding does not correlate with clinical potency[49]. Similarly, although the butyrophenones and related neuroleptics are reasonably active ($IC_{50} \sim 10$ μM) at inhibiting GABA uptake [48] or ^{3}H-naloxone binding to the opiate receptor[50] it is unlikely that either of these properties is the main determinant of their antischizophrenic activity. Their affinity is about 1000-fold lower in these systems than at the dopamine receptor ^{3}H-haloperidol binding site and when phenothiazine affinity for GABA uptake sites or opiate receptors is considered as well there is a poor correlation with clinical efficacy[48].

Although the neuroleptics are potent inhibitors of α-receptor binding, their relative activity differs markedly from their relative influences in inhibiting ^{3}H-haloperidol binding. For instance, spiroperidol is the most potent neuroleptic in competing for dopamine receptor binding but is one of the weaker agents in inhibiting α-receptor binding. Droperidol is one-fourth as potent as spiroperidol at the dopamine receptor but 26 times more potent than spiroperidol at the α-receptor[44].

As an index of α-receptor antagonism neuroleptics are often screened for their ability to antagonize the lethal effects of norepinephrine and epinephrine administered intravenously[20,44]. The fatal α-adrenergic vasopressor activity of norepinephrine and epinephrine is blocked by α-antagonists such as the ergot alkaloids, phentolamine and phenoxybenzamine but not by β-antagonists or other drugs[51]. Similar to its stereoselectivity in inhibiting WB-4101 binding, butaclamol is also stereospecific in antagonizing epinephrine-induced mortality[14]. The pharmacological relevance of WB-4101 binding sites is attested to by the high correlation between the potencies of drugs in competing for ^{3}H-WB-4101 binding and their potencies in blocking norepinephrine and epinephrine toxicity[44] ($r=0.94$, $p < 0.001$, and $r=0.88$, $p < 0.001$ respectively, animal data from Janssen[39]). This correlation across all neuroleptics is highly selective since there is no significant correlation between affinity for α-receptor binding sites and neuroleptic potency as measured in any dopamine receptor blocking tests such as catalepsy, amphetamine or apomorphine antagonism. Similarly there is poor correlation between neuroleptic affinity for ^{3}H-haloperidol binding sites and norepinephrine and epinephrine toxicity.

As therapeutic brain levels of neuroleptics may be expected to correspond to concentrations of the drugs which are required to obtain an optimal blockade of dopamine receptors, the clinical propensity of neuroleptics to block α-receptors would then not be related to their absolute

potencies as α-blockers, but to the ratio of their potencies as α-antagonists to their potencies as dopamine antagonists. Drugs with low ratios would be anticipated to elicit a substantial amount of α-adrenergic blockade at blood and brain levels of the drug required for adequate dopamine receptor blockade. By contrast, drugs with high ratios would be employed clinically at the very low dose levels required to secure dopamine receptor blockade and so, in general, would be less likely to elicit side effects associated with α-adrenergic blockade. Butyrophenones such as haloperidol and spiroperidol, and piperazine phenothiazines such as fluphenazine and trifluoperazine have a relatively low propensity to elicit hypotension and sedation and display ratios (K_i WB/K_i haloperidol) greater than 10. By contrast, promazine and clozapine the neuroleptics with the greatest tendency to cause orthostatic hypotension and sedation have ratios less than 0.2. In general, the finding that alkylamino and piperidine phenothiazines are more sedating and hypotensive than the piperazine agents is manifested in the lower ratios (K_i WB/K_i haloperidol of the former drugs). In general the butyrophenones tend to be relatively nonsedating and to have a low incidence of orthostatic hypotension and have substantially higher ratios[44]. The ratio of potencies of neuroleptics *in vivo* in blocking norepinephrine toxicity to their potency in blocking amphetamine-induced stereotypy behavior has been used as a measure of their relative propensity to cause autonomic sympatholytic side effects[20]. The ratio of drug potencies in these *in vivo* tests correlates highly ($r=0.90$, $p < 0.001$) with the ratios of drug potencies in competing *in vitro* for ^{3}H-WB-4101 and ^{3}H-haloperidol binding. The slope of the correlation line is about 1.0 suggesting that differential pharmacokinetic properties play a negligible role in determining the relative potencies of neuroleptics at their molecular sites of action after *in vivo* administration[44].

In conclusion studies of the dopamine receptor have demonstrated unequivocally that neuroleptic drugs, such as the phenothiazines and butyrophenones, do indeed block dopamine receptors and that their affinities for these sites correlate impressively with their *in vivo* pharmacological and clinical potencies. Such *in vitro* screening of new psychotropic drugs for dopamine receptor affinity may be predictive of antipsychotic activity and ability to produce tardive dyskinesia following chronic treatment. By comparing the ability of the same drug to interact with the muscarinic cholinergic and norepinephrine α-receptors in *in vitro* binding studies it may also be possible to predict its propensities for extrapyramidal and sympatholytic side effects. Such *in vitro* receptor binding methods may thus provide inexpensive, quick and effective screens for potent neuroleptic agents with a lowered propensity for unwanted side effects.

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Chapter 26: Biochemical Aspects of Muscular Disorders

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Progressive Muscular Dystrophy - The muscular dystrophies are the most common of all the muscular disorders. Extensive biochemical studies have focused on skeletal muscle in these diseases, especially the childhood (Duchenne) type, and various abnormalities were detected, but the causes of the diseases are still unknown.[1] Recent advances on the etiology of muscular dystrophy were reviewed by Peter[1] and Rowland.[2] Much attention has been directed to the muscle cell membrane, because the earliest sign of muscular dystrophy (especially the Duchenne type) is the increase of serum enzymes leaked from affected skeletal muscle. Hence an abnormality of muscle cell membrane is suspected as a primary defect in this disorder. Transport of calcium is abnormal in certain internal membranes (sarcoplasmic reticulum) but abnormalities are also detected in the surface membrane itself.[3,4] Membrane changes are not restricted to muscle cell in Duchenne dystrophy. Biochemical abnormalities in enzymatic activity,[5,6,7] (stimulation of ghost ATPase activity by ouabain), as well as abnormal lipid composition[8] and morphological changes[9] in red cells were reported and there were some controversies.[10,11] Recently interest was again focused on red blood cell membrane in Duchenne dystrophy. The data strongly suggest that Duchenne dystrophy should be considered a multisystemic disease of cell membranes.[12-16] Membrane abnormalities were also observed in myotonic dystrophy[14,15] and also in dystrophic mice and chickens.[14]

Specific insulin receptors were identified in skeletal muscle cell membrane and obese rats were shown to have fewer receptors than lean animals.[17] Interestingly, Duchenne patients usually become obese and likewise, of possible pertinence is the abnormal glucose metabolism observed in myotonic dystrophy patients.[18]

Concerning the pathogenesis of Duchenne muscular dystrophy, the neurogenic and vascular theories were proposed a few years ago and heated discussions ensued.[19,20] These theories are now waning.

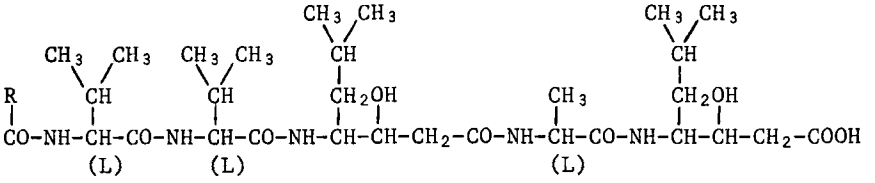
Administration of imipramine plus serotonin to rats resulted in muscle changes which were consistent with the vascular theory of Duchenne dystrophy.[21] However, other data suggest that the effect of the drugs on skeletal muscle were not due to the blockade of neuronal uptake of serotonin and subsequent vascular induced ischemia, but rather reflected a direct toxic effect on skeletal muscle.[22]

Motor endplates in Duchenne dystrophy show no adequate morphological evidence to support the neurogenic theory.[23] Muscle spindle innervation appeared normal in Duchenne dystrophy.[24]

Dystrophic animals are used to investigate the pathogenesis of human dystrophy. Transplantation of muscle[25] and cross-innervation[26] were studied and the dystrophies of mice and chickens were considered not to be of nervous origin.[26-28] However, there were papers suggesting metabolic changes in nerve,[29] neuromuscular junction[30] and endplate region[31] of dystrophic mice. "Functional denervation" was suggested in dystrophic mouse muscle.[32] It is important to note that the so-called muscular dystrophies in animals differ substantially from human dystrophy. Animal models must be chosen with care in any studies of putative therapeutic agents.

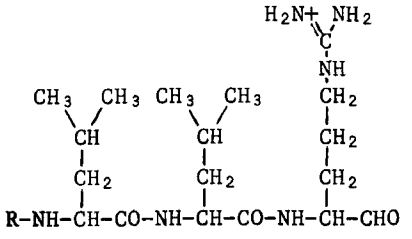
Muscular dystrophy of the Duchenne type is an incessantly progressive disease. Various drugs have not halted this progression.[33] The attempts are still continuing. Diethylstilbesterol was administered orally to Duchenne patients and creatine phosphokinase (CPK) in serum was decreased.[34] Experimentally, this drug reduced the enzyme efflux from mouse skeletal muscle. Prednisone sometimes decreases serum CPK and alleviates clinical symptoms temporarily and was suggested to be a therapeutic drug[35] in contrast to previous results.[36] However, transient or equivocal benefit is not sufficient and the evaluation must be cautious.[37] Proteinase inhibitors, e.g. pepstatin (1), leupeptin (2), and antipain (3) were shown to be effective against lysosomal cathepsins as well as other proteinases. The inhibitors appeared to delay atrophy and degeneration of dystrophic muscle fibers.[38] Proteinase is considered to play a role in muscle degeneration. The enzyme is presumably under neural control.[39] These experiments offer some prospects for probes at treatment of muscular dystrophy and degenerative diseases of muscle (see last section also).

1 - Pepstatin



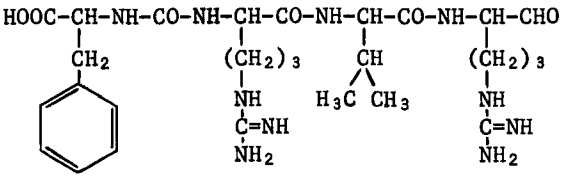
R = CH_3- $(CH_3)_2CH$-CH_2CH_2- etc.

2 - Leupeptin



R = CH_3CH_2CO- or CH_3CO-

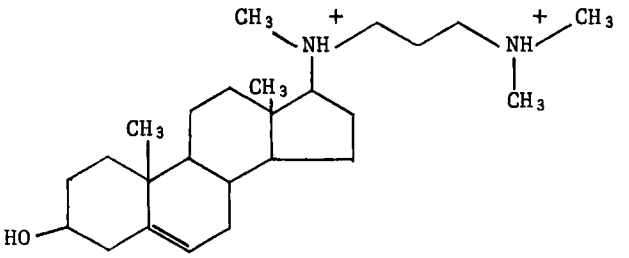
3 - Antipain



Myotonias - One form of human myotonia appears to reflect decreased chloride conductance in skeletal muscle.[40] Similarities and differences between human myotonia and drug-induced myotonia in rats were investigated and three possible mechanisms for decreased chloride conductance which might account for certain types of genetically determined and drug-induced myotonias were suggested.[41,42] No appreciable changes were observed morphologically in sarcolemma, sarcoplasmic reticulum or tubular system, which could explicitly explain the pathogenesis of myotonia.[43] Studies of ion transport by certain antibiotics may be important considerations in any therapeutic probes at treatment for certain myotonias.[41]

To investigate the mechanism of certain myotonic reactions, drug-induced myotonias in animals are studied. In 20,25-diazacholesterol (4) induced myotonia intracellular sodium was shown to be decreased and the changes in muscle were considered to be the result of a decreased membrane permeability for sodium.[44] No alteration in serum or red cell Na, K, Cl and (Na+K)-ATPase was detected. Diazacholesterol alters the electrical and mechanical properties of denervated muscle.[45] Myotonia was also seen in chickens and pigeons given water containing 3% KI for 1-10 days.[46] Significant rise in the concentration of a protein presumed to be α-actinin was demonstrated in the drug-induced myotonic rats.[47]

4 - 20,25-Diazacholesterol



Myotonic dystrophy shows not only myotonia, but also dystrophic changes of muscle and other multi-system involvement. In this disease membrane abnormalities were also detected in muscle[48,49] and erythrocytes.[41,48]

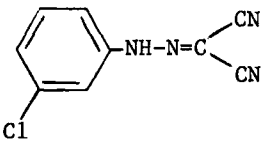
Mitochondrial Myopathy - Some degree of mitochondrial abnormality has been reported in several diseases of muscle. These morphological changes might be a response to some non-specific abnormal metabolic state of skeletal

muscle.

Luft et al[50] reported a case of severe hypermetabolism of non-thyroid origin and a defect in the maintenance of mitochondrial respiratory control. DiMauro et al[51] isolated mitochondrial fractions from such a patient and studies of oxidative phosphorylation showed defective respiratory control and normal phosphorylation capacity (loose coupling). The rate of energy-dependent calcium uptake by isolated mitochondria was normal, but the amount of calcium accumulated was much decreased. Calcium could not be retained and was spontaneously released into the medium within 30 seconds. "Recycling" of calcium between mitochondria and cytosol may take place in vivo and result in sustained stimulation of respiration and loose coupling.

A slowly progressive congenital neuromuscular disorder was reported in which the respiratory chain-linked energy transfer at a level common to all three energy coupling sites of respiratory chain was defective.[52] Uncouplers of mitochondrial oxidative phosphorylation (2,4-dinitrophenol and carbonylcyanide-m-chlorophenylhydrazone) (5) produced mitochondrial myopathy in rats.[53]

5 - Carbonylcyanide-m-chlorophenylhydrazone



McArdle's Disease - McArdle's disease is caused by the deficiency of phosphorylase in skeletal muscle. Two different types are known. In one the enzyme is lacking biochemically and immunologically, and the other has phosphorylase protein which is enzymatically inactive. Two different forms can be distinguished by the presence or absence of a protein subunit corresponding to phosphorylase in muscle extracts analyzed by SDS polyacrylamide gel electrophoresis.[54] Both types are known to be inherited as an autosomal recessive trait. Chui and Munsat[55] reported the disease transmitted dominantly through four generations. Myophosphorylase activity was absent histochemically and biochemically. Nearly all of the known hereditary enzyme disorders are recessive conditions, manifesting themselves only in homozygous individuals. Phosphorylase deficiency of autosomal dominant type appears to challenge the dictum that enzyme defects are characterized by recessive inheritance.

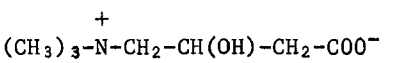
Ischemic exercise is usually used for the diagnosis of McArdle's disease and the lack of venous lactate increase is believed to be the sine qua non for the diagnosis. In a patient described by Sahn and Magee[56] venous serum lactate increased in the arm after isometric and isotonic ischemic exercise, although the increase was significantly less than in a normal control. Histochemical reactions and biochemical assays confirmed the absence of phosphorylase. Although the symptoms were

different from the usual McArdle's disease, this condition is considered to be the same disease.

Late-Onset Acid Maltase Deficiency - This disease, originally described by Hudgson et al[57] in 1968, presents clinically truncal and proximal muscle weakness. This disorder is often misdiagnosed as a limb-girdle type of muscular dystrophy or as polymyositis. (For review of inflammatory myopathies, see reference 58). Acid maltase activity is deficient in biopsied skeletal muscle, but was normal or only partially deficient in leukocytes. Daily urinary excretion of this enzyme is significantly decreased in the patients.[59] This provides a simple and reliable method for the detection of patients deficient or partially deficient of acid maltase. Moreover, the marked decrease of the enzyme activity in urine suggested that tissues other than muscle, liver and fibroblast in culture might manifest the enzyme defect in late-onset acid maltase deficiency.[59] Indeed, muscle cultured from a patient with late-onset acid maltase deficiency showed the same morphological and biochemical abnormalities characteristic of biopsied muscle.[60] Although the normal role of acid maltase in muscle is not known, glycogen trapped within lysosomes might act like non-metabolizable material and elicit lysosomal proliferation. A ketogenic diet has been suggested to slow down the accumulation of glycogen.[61]

Carnitine Deficiency - Carnitine (6) deficiency of skeletal muscle associated with lipid storage myopathy was reported by Engel and Angelini[62] in 1973 as a new syndrome. Since their report about 10 other cases have been reported. Angelini et al[63] described a 10-year-old girl with this disease who was treated with 2.0 g l-carnitine per day and with a medium-chain triglyceride diet. She showed a rapid improvement with recovery of strength. A muscle biopsy eight months later showed a decreased lipid content. Oral carnitine replacement is an effective treatment for this disease.[63]

6 - Carnitine



Probes at Replacement Therapy for Enzyme Deficiency Diseases - Reports on use of immunoglobulin-coated liposomes to introduce enzymes into certain cells in vitro are promising probes at definitive treatment of deficiency diseases.[64,65] These approaches have potential application to other diseases in which novel methods of packaging must be designed to protect the therapeutic agent from the immune surveillance system and other systems expected to interfere with these and other replacement therapies for which the need is enormous.

Other vehicles for enzyme replacement in deficiency diseases might include transfusion of cells loaded with the deficient enzyme or whole tissues (e.g. kidney). A major problem in all of these approaches will be the exclusive delivery of the "cure"-containing vehicles to the proper target site. This presumably will await modeling of the vehicle in such a way as to permit its recognition (via specific receptors) and ingestion by

the deficient tissue(s). Other putative avenues to "definitive" treatment of diseases characterized by deficiencies of macromolecules will, of course, include 1) correction of deficiencies by transplantation of cells (e.g. fibroblasts) from normal, compatible donors and 2) activation or manipulation of small amounts of residual enzyme activities or alternative pathways to minimize the deleterious effects of deficiency diseases which lead to tissue damage by excess storage of substrate or deficient availability of product.[66]

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Section VI - Topics in Chemistry

Editor: R. E. Counsell, University of Michigan, Ann Arbor, Michigan

Chapter 27. Reactions of Interest in Medicinal Chemistry

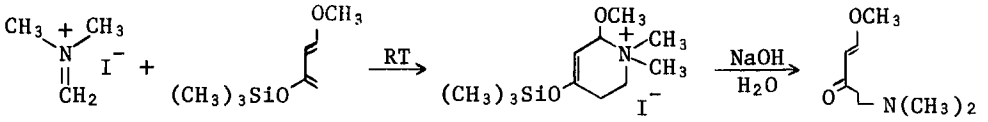
David M. Spatz, Dow Chemical U.S.A., Pharmaceutical R&D, Midland, MI

Useful reference books published recently include: Annual Reports in Organic Synthesis-1975, by R. B. Miller and L. G. Wade, Jr.; Transition Metal Organometallics in Organic Synthesis, edited by H. Alper; Survey of Organic Syntheses, Volume 2, by C. A. Buehler and D. E. Pearson; Organic Synthesis, Volume 55, edited by S. Masamune; Organic Reactions, Volumes 23 and 24, edited by W. G. Dauben; Advances in Heterocyclic Chemistry, Volume 19, edited by A. R. Katritzky and A. J. Boulton; Synthetic Methods, Volume 30, edited by W. Theilheimer; Reagents for Organic Synthesis, Volume 5 by M. Fieser and L. M. Fieser; Special Topics in Heterocyclic Chemistry, Volume 30, edited by A. Weissberger and E. C. Taylor; Advances in Organometallic Chemistry, Volume 14, edited by F. G. Stone and R. West.

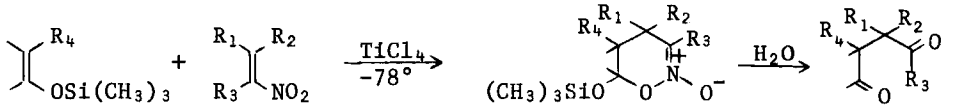
Reviews - Acyl anion equivalents,[1,2] crown ethers in synthesis,[3] intramolecular 1,3-dipolar additions,[4] oxazolines in synthesis,[5] oxidation-reduction condensations,[6] oxythallation,[7] transition metals in synthesis,[8,9] and ynamines in synthesis.[10] Also reviewed are syntheses of pyridines,[11] pyrroles,[12] and indolizines.[13] Other reviews are in specific sections. The Institute for Scientific Information now publishes Index to Scientific Reviews semi-annually.

C-C Bond Formations - Organoboranes for C-C bond formation have been reviewed.[14]

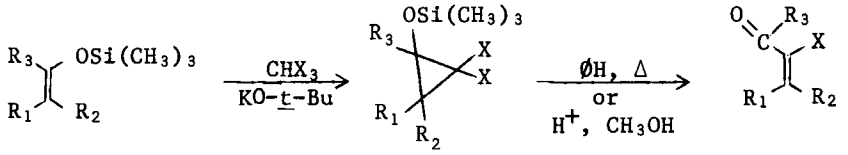
Trimethylsilyl enol ethers continue to be useful synthons for various aldol type[15,16] and Michael[17,18] reactions. Their utility in part is due to their ease of regiospecific preparation, ease of cleavage and high reactivity. Danishefsky and coworkers have shown that silyl enol ethers react with dimethyl(methylene)ammonium iodide yielding Mannich bases.[19] Otherwise inaccessible Mannich bases are accessible via the series below.



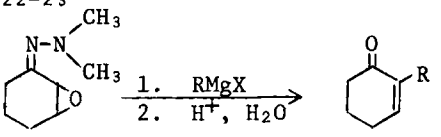
Nitro-olefins react with trimethylsilyl enol ethers under Lewis acid catalysis in a regiospecific manner yielding 1,4-diketones.[20]



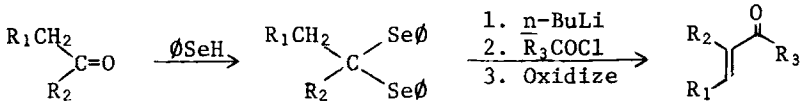
α-Halo-α,β-unsaturated carbonyl compounds are conveniently prepared by addition of a dihalo carbene to silyl enol ethers.[21] Further uses of trimethylsilyl enol ethers are discussed in later sections.



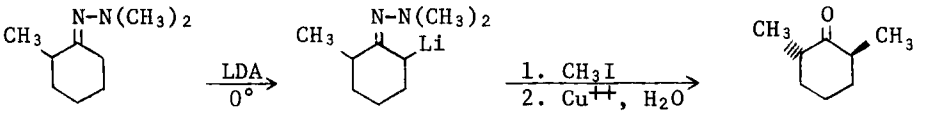
Substitution in the α-position of an α,β-unsaturated ketone can be achieved by addition of an alkyl or aryl Grignard reagent to an α,β-epoxy N,N-dimethyl hydrazone.[22-23]



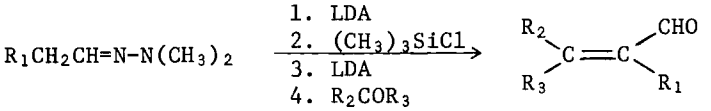
α-Seleno carbanions added to the appropriate electrophile yield an α-seleno carbonyl compound, which upon oxidation produces α,β-unsaturated ketones.[24]



Corey and coworkers have outlined the wide usefulness of N,N-dimethyl hydrazones (DMH) in synthesis.[25-9] The parent carbonyl compounds can be regenerated by oxidative hydrolysis via periodate at pH 7 or with the very mild cupric acetate in water-THF. Metallation of these hydrazones selectively occurs at the less alkylated carbon. Alkylation occurred axially in the cyclohexane derivatives studied. Quenching of the metallated DMH's

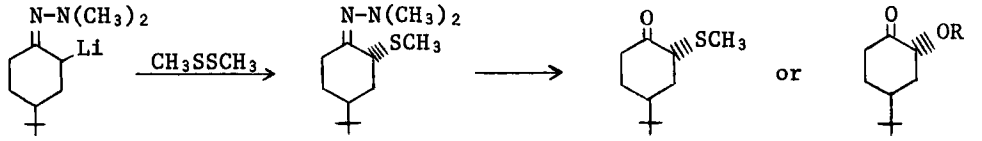


with carbonyl compounds or epoxides yields β-hydroxycarbonyl compounds or γ-hydroxycarbonyl compounds, respectively. The metallated DMH's add 1,2- to α,β-unsaturated carbonyl compounds; however, cuprate derivatives can be formed which add 1,4 and afford a path to 1,5-diketones. Silyl aldehyde DMH's produce α,β-unsaturated aldehydes.



(LDA = Lithium Diisopropylamide)

Another interesting α-functionalization of the metallated DMH's is achieved by addition of dimethyl disulfide. Again, the new group is added axially. Hydrolysis produces 2-thiomethylketones. Alternatively, an axial acetoxy or methoxy group is introduced by mercuric chloride catalyzed solvolysis in acetic acid or methanol.



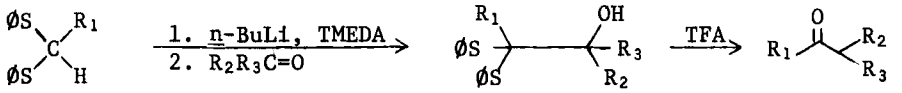
Enantioselective alkylation of cyclohexanones can be accomplished via a chiral lithio-chelated enamine.[30]

Doubly lithiated nitroalkanes react rapidly with electrophiles providing an improved method for C-C bond formation from nitroalkanes.[31]

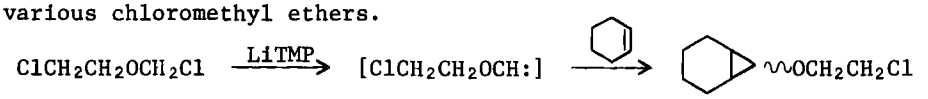
Doubly lithiated methanol can be prepared to give a nucleophilic hydroxymethylation reagent.[32]

$$\text{ØCHO} + \text{LiCH}_2\text{OLi} \longrightarrow \text{ØCH(OH)-CH}_2\text{OH}$$

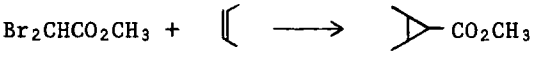
The bis(phenylthio)carbanion can be condensed with a ketone yielding an intermediate bis(phenylthio)alcohol.[33] Trifluoroacetic acid catalyzed hydrolysis generates an alkylated carbonyl compound without the utilization of an alkyl halide.



Olofson and coworkers have published improved syntheses of cyclopropanols.[34-6] These make use of the high selectivity of lithium 2,2,6,6-tetramethylpiperidide (LiTMP, H$^+$arpoon) in generation of alkoxy carbenes from various chloromethyl ethers.



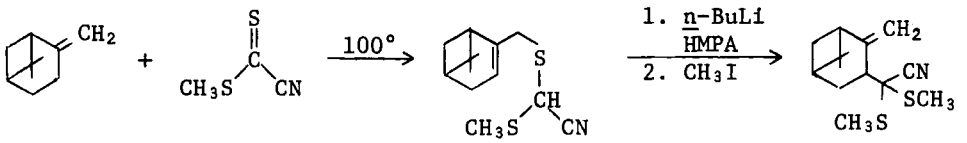
Cyclopropanes can be synthesized from many organic gem-dihalides using copper and a trace of iodine as catalysts.[37] This procedure is comparable to the Simmons-Smith reaction in yields and is more convenient because of a wider range of substrates available.



Several improved titanium-derived catalysts have been reported for the reductive coupling of carbonyls to diols and olefins, activated halides to alkanes and vic-dihalides to olefins.[38-40]

Allenes are cleanly formed by addition of Grignard reagents to propargyl chlorides with ferric chloride catalyst.[41]

Formation of a functionalized C-C bond in an allylic position can be carried out with methyl cyanodithioformate via a sequence of an ene reaction followed by a [2,3]-sigmatropic rearrangement.[42]



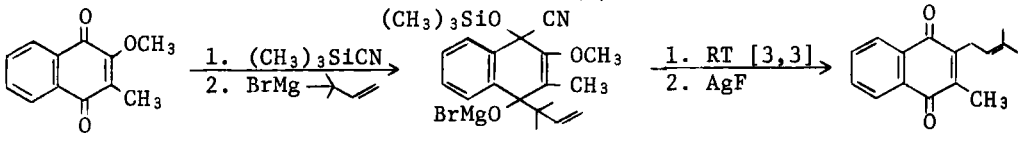
Interesting new 1,3-dienes for the Diels-Alder reaction include (Z)-1-phenylthio-2-methoxy-1,3-butadiene,[43] 2-methoxy-3-phenylthio-1,3-butadine,[44] and N-acyl-1-amino-1,3-butadiene.[45]

Aromatic Substitution - Phenols can be oxidized directly to ortho-quinones with diphenylseleninic anhydride.[46] Blocking of the para-position is unnecessary and yields are good.

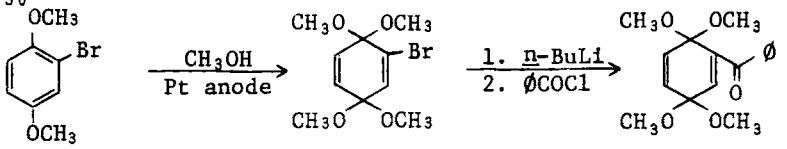
A trifluoromethylthio group can be introduced into an aromatic ring by coupling of trifluoromethylthio copper (I) and an aryl bromide or iodide.[4] Aryl chlorides are inert.

Nucleophilic displacement of the nitro group of nitrobenzenes substituted with a variety of electron-withdrawing groups is readily possible at 25°C with HMPA as solvent.[48] Carbon, oxygen, and sulfur nucleophiles have been used with excellent yields reported.

Regiospecific quinone isoprenylation can be effected by the following scheme used in the synthesis of vitamin $K_{2(5)}$.[49]

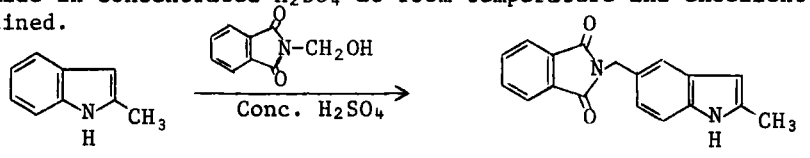


A sequence of anodic oxidation followed by metallation provides quinone carbanions which allow specific electrophilic substitution of quinones.[50]

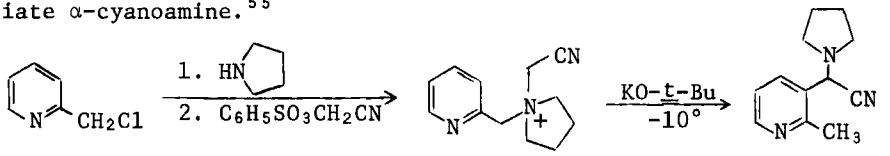


Heterocycles - Aromatic substitution via N-oxides has been reviewed.[51] Palladium catalyzed intramolecular addition of amines to olefins has resulted in new syntheses of indoles[52-3] and isoquinuclidines.[53]

An interesting reaction for 5-amidomethylation of indoles unblocked in the 3-position has been reported.[54] The conditions are N-hydroxymethylphthalimide in concentrated H_2SO_4 at room temperature and excellent yields are obtained.

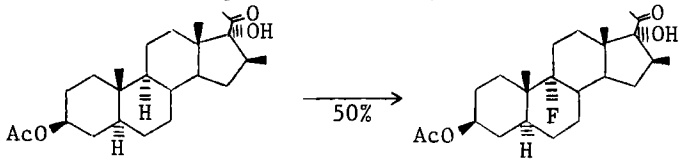


A general synthesis of 2-alkyl-3-acylpyridines is achieved from 2-substituted pyridines via a [2,3]-sigmatropic rearrangement of an intermediate α-cyanoamine.[55]



Halogenations - An improved procedure for the regiospecific preparation of α-bromocarbonyl compounds uses the requisite trimethylsilyl enol ether and either bromine or N-bromosuccinimide.[56]

Direct fluorination at saturated carbon is possible with either elemental fluorine or CF_3OF and a radical inhibitor.[57-8] The transformation is an electrophilic fluorination, regiospecific by virtue of the highly polar transition state sensitive to the inductive effects of nearby or remote polar substituents. Substitution occurs almost exclusively at tertiary positions with monosubstitution predominating. With various substituents, selective fluorination at positions 9, 14, or 17 of the steroid nucleus was reported.



Primary unbranched amines have been converted to geminal dihalides by the combination of alkyl nitrites and anhydrous copper (II) halides.[59]

$$\phi CH_2CH_2CH_2NH_2 \xrightarrow[t\text{-BuONO}]{CuBr_2} \phi CH_2CH_2CHBr_2$$

Alkyl halides can be prepared from selenides and selenoxides.[60]

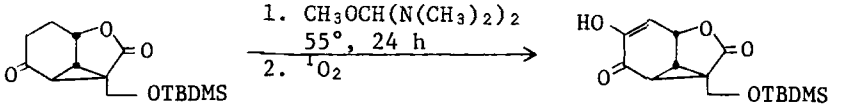
Oxidations - Active manganese dioxide oxidations have been reviewed.[61]

Several new procedures for the selective oxidation of secondary alcohols in the presence of primary alcohols have appeared. Distannoxane-bromine, a neutral reagent, efficiently oxidizes secondary or benzylic alcohols to ketones in the presence of primary alochols.[62] Trialkyltin alkoxides and bromine oxidize both primary and secondary alcohols.[63] Woelm W-200, neutral, dehydrated alumina oxidizes secondary alcohols to ketones in the presence of primary alcohols with trichloroacetaldehyde as the hydride acceptor.[64] The reverse reduction process had previously been reported. Oxidation of triphenylmethyl ethers of primary, secondary diols with triphenyl carbenium salts proceeds only at the secondary positions.[65]

Dimethylsulfoxide trifluoroacetic anhydride at low temperature is a mild reagent for the oxidation of hindered alcohols.[66-7]

γ-Hydroxylation of α,β-unsaturated esters is possible via α-thiomethylation, oxidation and [2,3]-sigmatropic rearrangement.[68]

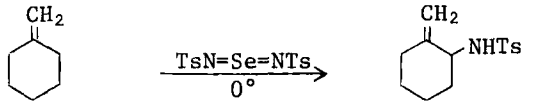
α-Diketones are prepared in excellent yield from ketones by singlet oxygen oxidation of an intermediate enamino ketone.[69]

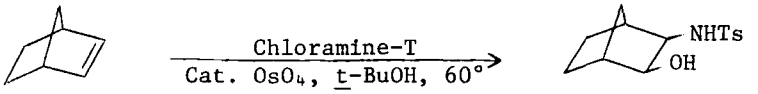


Regiospecific preparation of α-benzoyloxy carbonyl compounds by lead tetrabenzoate (LTB) oxidation of the trimethylsilyl enol ethers is possible.[70] Similarly, α-thiolated ketones can be prepared from a disulfide.[71]

Sulfoxides can be obtained from sulfides without sulfone formation by either acyl nitrates (ØCOCl + HNO_3 or Ac_2O + HNO_3)[72] or sulfuryl chloride and wet silica gel.[73] Both methods are clean, mild and rapid.

Sharpless and coworkers have reported a new series of reagents for allylic amination of olefins,[74-5] vicinal oxyamination of olefins,[76] and 1,2-diamination of 1,3-dienes.[77]





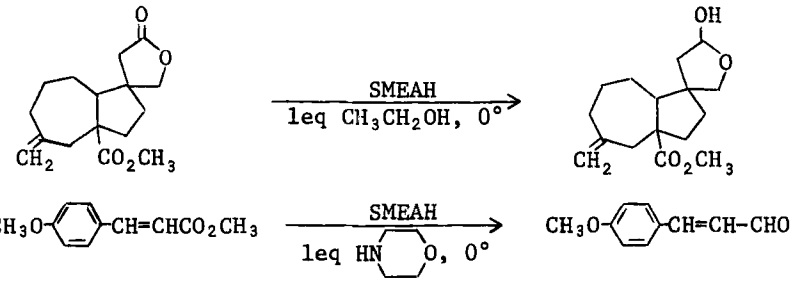
Reduction - An excellent review of diborane reductions has appeared.[78]

Lithium and potassium tri-sec-butylborohydride (L- and K-Selectride) selectively reduce the olefin of many α,β-unsaturated carbonyl compounds.[79] The intermediate enolate anions may also be trapped by electrophiles, providing a regiospecific reductive alkylation of α,β-unsaturated carbonyl compounds.

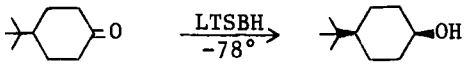
Sodium cyanoborohydride in acidic methanol reduces α,β-unsaturated esters, nitriles, and nitro compounds to their saturated derivatives.[80]

The olefinic bond of α,β-unsaturated carbonyl compounds can also be reduced by two transition metal hydrides, $NaHFe_2(CO)_8$[81] and $NaHCr_2(CO)_{10}$.[82] Neither reagent reduces nitriles, ketones, aldehydes or non-conjugated carbon-carbon double bonds.

Sodium bis[2-methoxyethoxy]aluminum hydride (SMEAH) can be modified to selectively and rapidly reduce lactones to lactols or esters to aldehydes.[83]



Lithium trisiamylborohydride (LTSBH) shows unique stereoselectivity in the reduction of unhindered ketones.[84] Ketones such as 4-t-butylcyclohexanone undergo exclusive equatorial attack yielding the cis-carbinol. The reagent is superior to LTMBH and L-Selectride.



Protecting Groups - Detonation has been reported for compounds where oxidation of tetrahydropyranyl (THP) ether derivatives was performed.[85] It appears that the THP-protecting group forms sensitive organic peroxides when treated with peroxy reagents. Normal precautions in the work-up of such reactions were insufficient in destruction of the sensitive compounds. THF, dioxolane, and methoxymethyl ether groups should also be presumed to form explosive derivatives upon contact with peroxy reagents.

Aldrich now offers 2-(t-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON) as a non-explosive replacement for the thermally unstable and shock-sensitive t-BOC azide.[86]

The β-methoxyethoxymethyl (MEM) group has been introduced as a new general protecting group for the hydroxyl function.[87] The MEM group is rapidly introduced under either aprotic basic or aprotic neutral conditions. The MEM group may be selectively cleaved with $ZnBr_2$ or $TiCl_4$ with no interference from esters or benzyl, allyl, THP, TBDMS, trichloroethyl, or MTM ethers. Conversely, these groups may be removed in the presence of MEM ethers. MEM ethers are stable to strong bases, reducing agents, organometallic reagents, many oxidizing agents, and mild acids.

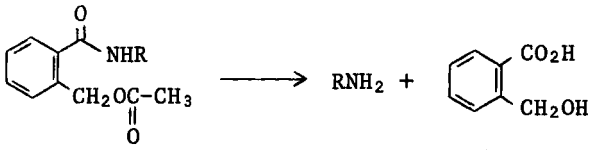
Further extensions of the use of the methylthiomethyl (MTM) protecting group for alcohols have been reported.[88-9]

Methyl ethers can be cleaved in minutes at room temperature with diiodomethyl methyl ether.[90]

Methylenation of catechols is greatly improved with potassium or cesium fluoride in DMF and CH_2Cl_2 or CH_2Br_2.[91]

The S-p-methoxybenzyl and S-t-butyl protecting groups for cysteine are rapidly cleaved with mercury (II) trifluoroacetate.[92]

2-Acyloxymethylbenzoic acids can be used to protect amines.[93] The amides so derived can be cleaved with mild acid or base due to neighboring group participation of the generated alcohol.



The trimethylsilyloxy group of allylic or benzylic alcohols is readily displaced by nucleophiles (malonate anion, alkyl Grignard reagents, hydride ($AlCl_3$-$LiAlH_4$)).[94] This limitation as a protecting group should be noted.

Miscellaneous - Chiral and achiral thiiranes can be prepared from carbonyl compounds and 2-(alkylthio)-2-oxazolines.[95]

β-Ketoesters are mildly decarboxylated in refluxing 1.5% aqueous dioxane using alumina (Merck, type T, basic) as catalyst.[96]

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Chapter 28. Synthetic Applications of Metalated Carboxylic Acids

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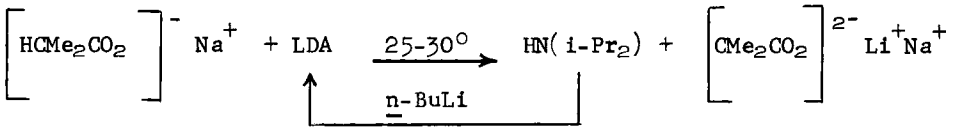
I. Introduction - Synthetic chemists must consider not only techniques for selectively installing, blocking and manipulating essential functionality of target molecules but also methodology that allows selectivity and stereochemical control over the architecture of the carbon framework. Carbon bond forming reactions, particularly alkylation reactions, are fundamental tools used for this purpose. The degree of synthetic versatility that can be exercised during formation of a new bond depends as much upon the character of the carbanionic intermediate as it does upon the electrophile. The utility of metalated carboxylic acids in synthesis is derived from the ability of these dianions to react with most electrophiles. Unlike many carbanions, those derived from carboxylic acids display unusual stability so forcing conditions can be employed if necessary, and they appear to suffer few limitations in sterically crowded environments. Further, dialkylation is minimal, if it occurs at all, O-alkylation has not been observed, and, of course, blocking groups are unnecessary. As a result of these characteristics, metalated carboxylic acids can be used to advantage for transformations for which the malonic ester, Haller-Bauer and Reformatsky reactions have been employed traditionally. Even though most reactions reported for carboxylic acid dianions have been extended to anions of functional derivatives (esters, amides, nitriles, lactams and lactones), this review will restrict its scope to metalated carboxylic acids: formation, structural variations and synthetic applications. Reactions of Ivanov reagents[1] and closely related lithium reagents[2] have been reviewed adequately elsewhere. Likewise, the Reformatsky reaction (ester anions) has been reviewed recently.[3]

II. Formation of Metalated Carboxylic Acids - Several strong bases have been used to abstract protons from the α-carbon of carboxylate salts, but most require either strenuous conditions or prior preparation of the base. For example, sodium metal,[4] sodium amide,[5,6] naphthenyllithium,[7,8] amylsodium[9] and various substituted lithium amides[10] have been used for this purpose. Lithium diisopropylamide (LDA) was reported initially to be convenient[10] because it has high basicity, good solubility, substantial steric hindrance, and because diisopropylamine, from which it is derived, has favorable physical properties, low cost and general availability. However, more hindered lithium amides may be necessary for some purposes.[11] Loss of protons from the α-carbon of carboxylic acids is relatively slow and temperatures above 0° may be required to establish favorable metalation rates. The dianions formed are stable at least to the boiling point of tetrahydrofuran.

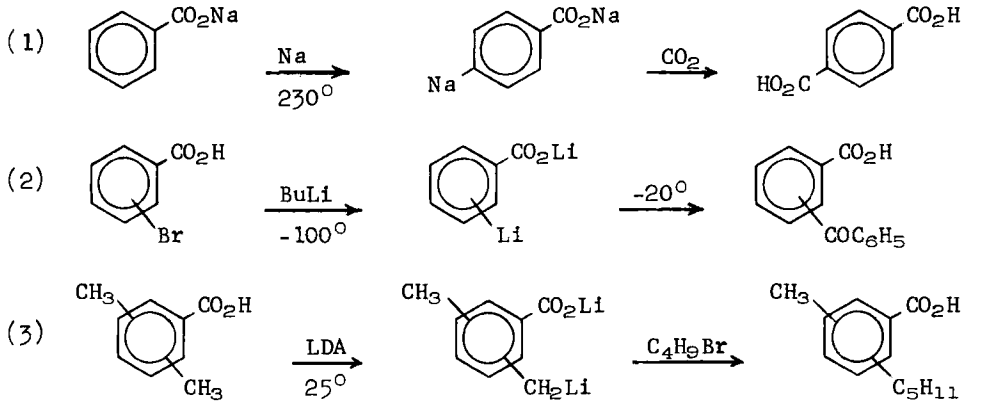
On a small scale, it is convenient to add the carboxylic acid to two equivalents of LDA[10] or other lithium amide, but larger experiments can be performed by addition of an available organolithium reagent to a suspension of a preformed salt containing equivalent or lesser molar amounts of the amine.[12,13] Diisopropylamine reacts more rapidly than carboxylate salts with n-butyllithium. When LDA so formed abstracts a proton from the carboxylate salt, the amine produced can react again with addi-

tional n-butyllithium. High yields of alkylation products can be obtained when the dianion is formed in mixtures containing as little as 3 mole percent of amine.[14]



Tetrahydrofuran (THF) is preferred as solvent.[14] Dimethoxyethane has been reported[15] to react with LDA and presumably other glycol based solvents could behave similarly. Such behavior could account for low yields experienced from their use in alkylation reactions.[14] In THF, dianions of disubstituted acetic acids usually form homogeneous solutions, while dianions of monosubstituted acetic acids form heterogeneous mixtures unless the substituent is bulky (t-butyl, phenyl) and both cations are lithium.[12,14] Hexamethylphosphoric triamide (HMP) has been recommended[16] as a cosolvent with THF for producing homogeneous solutions of metalated monosubstituted acetic acids when both gegenions are lithium, but its presence promotes elimination rather than substitution in alkylation reactions.

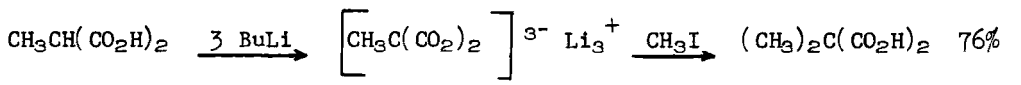
III. Structural Limitations - Few structural features have been reported which prevent dianion formation. Even benzoic acid can be metalated by proton abstraction[17] (eq 1) or by metal-halogen exchange[18] (eq 2). All three toluic acids form dianions, and dimethyl benzoic acids (eq 3) can be selectively metalated and alkylated.[19] Ortho, meta and para methyl groups of dimethylbenzoic acids react in the order: o>p>m.



Nearly all common mono- and disubstituted acetic acids, including several functionally substituted examples, have been reported to metalate. Unsaturated as well as saturated alicyclic carboxylic acids form dianions, but cyclopropane carboxylic acid is exceptional. Low yields of alkylated products are produced,[14,20] suggesting incomplete dianion formation.

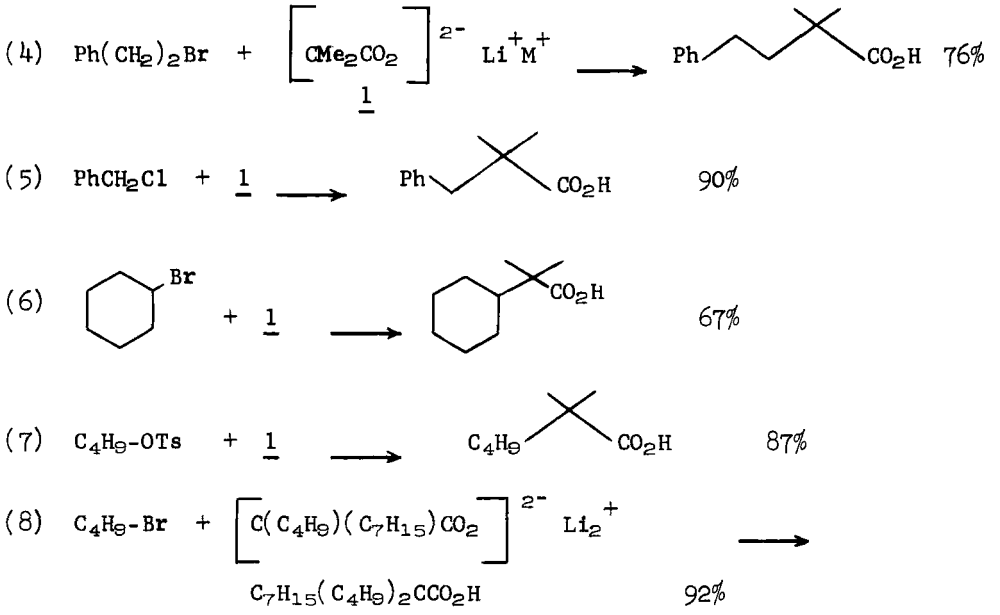
Grignard reagents and unsubstituted alkali metal amides are sufficently strong bases to remove protons from α, β- and β,γ-unsaturated carboxylic acids. Formation of dianions of unsaturated carboxylic acids was first demonstrated[21] by trapping the intermediates with methyl iodide. Subsequent isolation of the products revealed that α-substitution had occurred. More recently, a relatively large number of examples has been investigated.[22-24] Unsaturation is stable in structures in which the double bond is located at positions remote to the carboxyl group.[22,24]

Malonic acids[25] and malonic acid esters,[26] likewise, form carbanions which can be alkylated. Trianions of malonic acids can be formed by treatment with n-butyllithium in the absence of an amine.

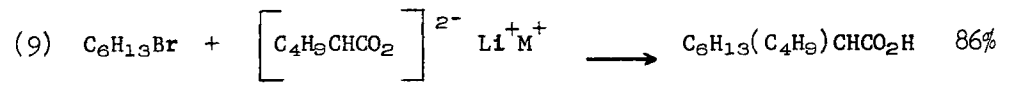


IV. Reactions of Metalated Carboxylic Acids - Metalated carboxylic acids react with most electrophiles if the dianion is associated with alkali metal cations. However, Cu(I) dianions[22] react readily only with activated allylic or benzylic halides.

A. Alkylation: 1. Alkyl Halides - Alkylation is, perhaps, the most useful synthetic reaction of metalated carboxylic acids. The reaction proceeds smoothly and it produces preparative yields of products for a variety of functionally different alkyl halides, including examples susceptible to elimination.[10] The reaction is not sensitive to the leaving group, nor is it sensitive to steric effects as the following examples[12,14,16] illustrate. The Haller-Bauer procedure fails where two of

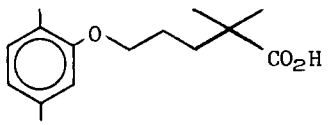


the α-substituents are larger than ethyl, and it is advantageous to install the largest substituents first.[27] As illustrated in eq 8, large, highly branched carboxylic acids can be prepared efficiently from metalated carboxylic acids, and sequential alkylation does not depend upon the order in which substituents are installed.[16] Dialkylation of monosubstituted acetic acids is negligible in most cases (eq 9).[13] Lower members of the homologous series of aliphatic carboxylic acids afford lower yields, and small amounts of dialkylated products are produced as a re-



(9) $C_6H_{13}Br + [C_4H_9CHCO_2]^{2-} Li^+M^+ \longrightarrow C_6H_{13}(C_4H_9)CHCO_2H$ 86%

sult of incomplete metalation caused by poor solubility of the carboxylate salts. This complication can be minimized by use of HMP as cosolvent[16] or by suitable cation changes.[28] 2-Substituted tetralones and 2-substituted indanones can be prepared readily if the products of eq 4 and 5 are cyclized.[14] Alkylation reactions of carboxylic acids performed with aryl haloalkyl ethers and α, ω-dihaloalkanes have practical significance. The products produced from these reactions have antihyperlipidemic properties,[29-37] and two drugs, gemfibrozil, 2, and gemcadiol, 3, have been studied clinically.



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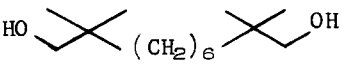
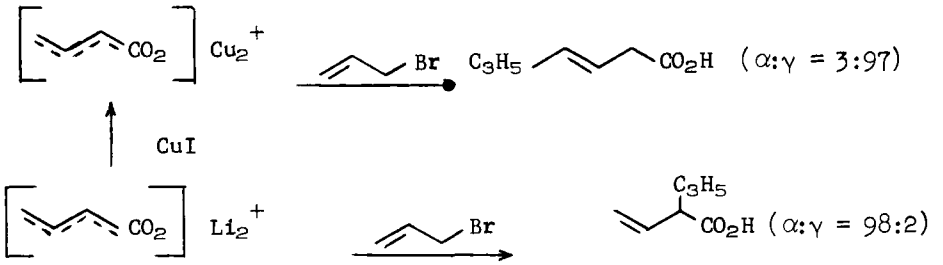
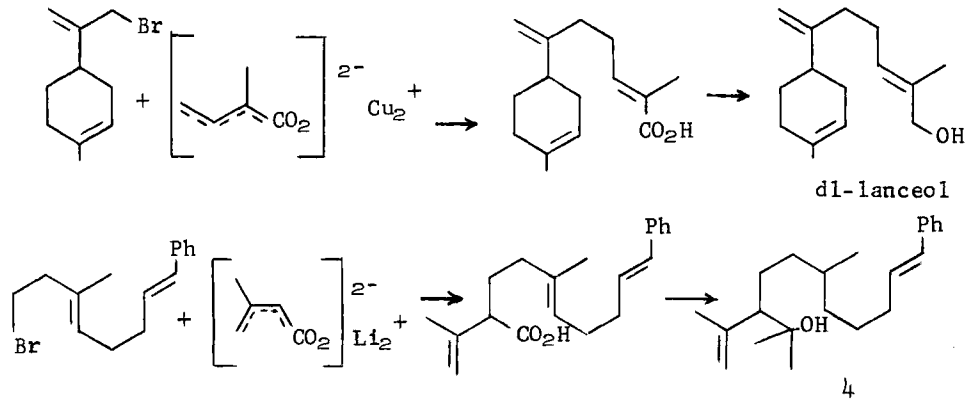


3

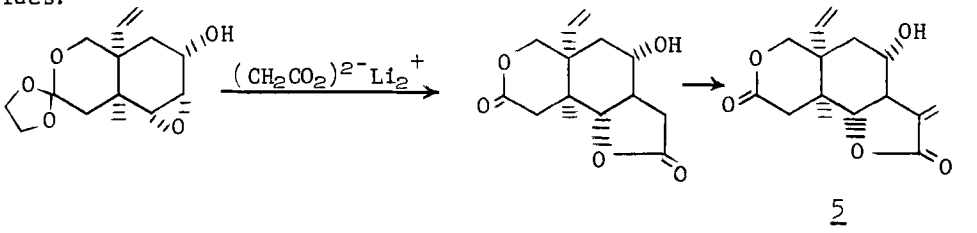
Dianions derived from α, β- or β, γ-unsaturated carboxylic acids alkylate at the α-carbon if the dianions are associated with alkali metal cations.[22-24] However, high proportions of γ-substitution are observed when Cu(I) dianions are employed.[22] The preparation of dlanceol illustrates synthetic use of γ-alkylation,[22] and the preparation



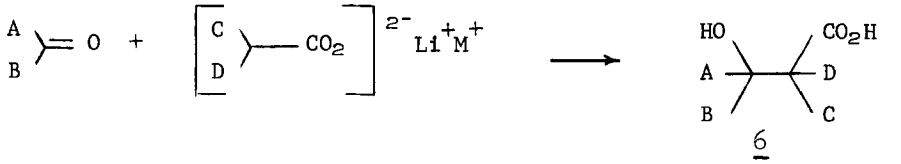
of polyene, 4, illustrates α-alkylation[38] of dianions of unsaturated carboxylic acids.



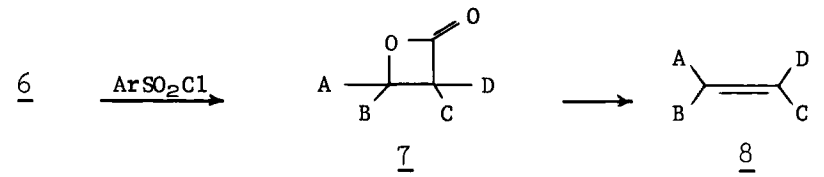
2. Epoxides - The first indication of the synthetic utility of metalated carboxylic acids resulted from efforts to prepare steroidal aldosterone inhibitors from spiroepoxides.[39] Model studies indicated that the reaction fails as a result of severe steric hindrance in either the epoxide or carboxylic acid and that monosubstitution occurs for the same reason.[39] The reaction has been used in a key step of an elegant synthesis of vernolepin, 5.[40] Forcing conditions are required, and, in contrast to acid dianions, anions of unactivated esters fail to react with epoxides.[39,41]



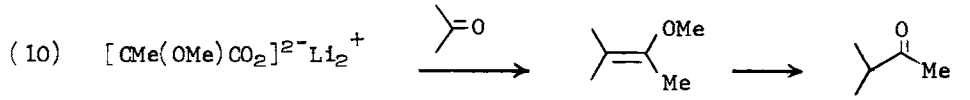
B. Addition to Carbonyl Compounds - Addition of metalated carboxylic acids to aldehydes and ketones affords a useful and versatile alternative to the Reformatsky reaction.[42] The reaction is sensitive to steric effects



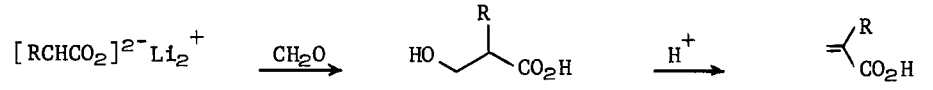
in both reaction partners, but the method has a real synthetic advantage because β-hydroxy acids are not otherwise readily accessible. Although α-adducts predominate, addition occurs to both faces of 17-keto steroids.[43] The reaction has resulted in a versatile and stereospecific olefin synthesis.[44] Isomers of 6 can be separated and degraded via β-lactones, 7, yielding hindered olefins, 8, of known stereochemistry. The reaction has been used to introduce acetyl substituents[45] (eq 10) and isopropylidene groups,[46] and it has been used for the preparation of



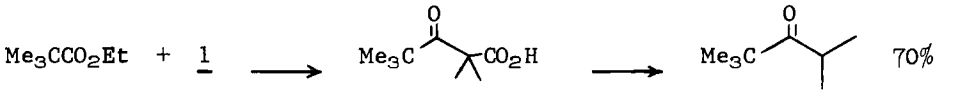
cycloalkylidene cycloalkanes.[47] This sequence should rival the Wittig reaction for many applications.



Substituted acrylic acids have been prepared by reaction of acid dianions with monomeric formaldehyde.[48] A variation of the reaction permits in situ formation of formaldehyde from anions of methoxymethyl esters.[49]



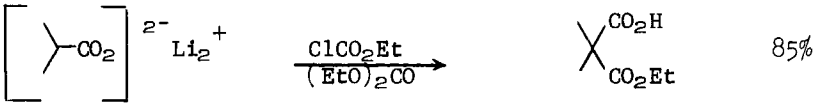
Esters react with metalated carboxylic acids yielding β-ketoacids from which aldehydes[50] and ketones[51] may be derived. Like the Adam olefin



synthesis, the dianion acts as a reactive carrier of the carbon fragment attached to the carboxyl group. In cases where the carboxyl function is eventually eliminated, metalated carboxylic acids can afford useful synthetic alternatives to Grignard and organolithium reagents. Unsaturated esters and nitriles react by 1,4-addition,[51] but unsaturated aldehydes undergo 1,2-addition.[42]

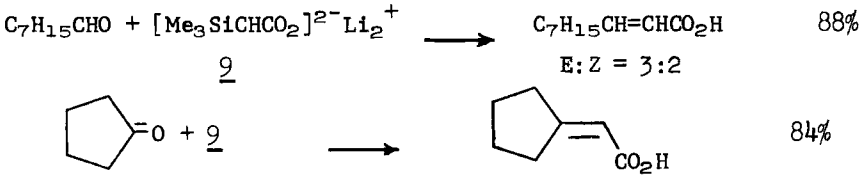
Carbon dioxide was the first electrophile demonstrated to react with metalated carboxylic acids.[9] The reaction has been used to measure the extent of metalation[23] and to prepare malonic acids which would be difficult to obtain by other methods.[52] Malonic acid esters are available

by treatment of acid dianions with carbon dioxide derivatives[53] or, equivalently, by carboxylation of ester anions.[54]

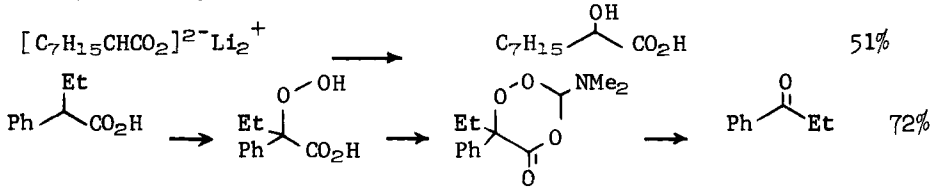


Trimethylsilylacetic acid forms adducts with carbonyl compounds which undergo spontaneous elimination.[55] The unsaturation produced appears solely in the α, β-positions, but E and Z isomers are formed in

approximately equal amounts. The reaction occurs efficiently with cyclopentanone as well. Unfortunately, the utility of this dianion is restricted by its limited accessibilility.

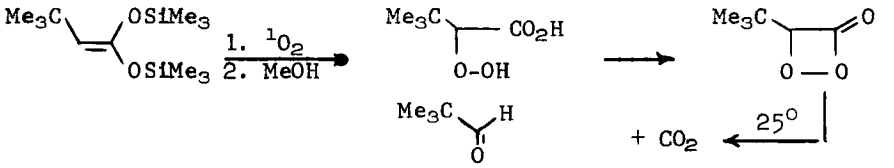


C. Oxygenation - Metalated carboxylic acids react instantaneously with oxygen, and, depending upon reaction and workup conditions, α-hydroxy-[56] or α-hydroperoxycarboxylic acids[57] are obtained. If low temperatures

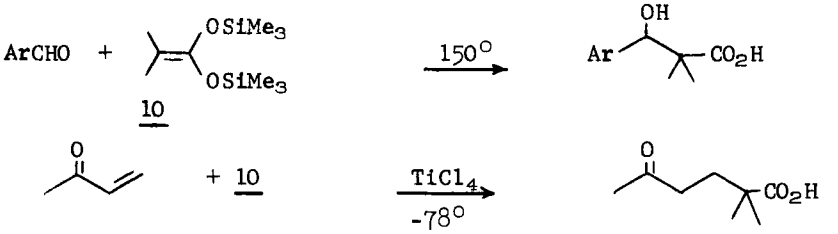


are employed, the hydroperoxide intermediate can be converted to a ketone by treatment with dimethylformamide dimethylacetal.

D. Silylation - Ketene bis(trimethylsilyl)acetals result from treatment of acid dianions with chlorotrimethylsilane.[58,59] Esters behave analogously.[60] Silylated ketene acetals can react like acid dianion equivalents. For example, reaction with singlet oxygen was used to produce the first α-peroxylactone.[61,62]



Non-enolizable aldehydes react with silylated ketene acetals by thermal reorganization,[63] but milder conditions may be used in the presence of titanium tetrachloride.[64]



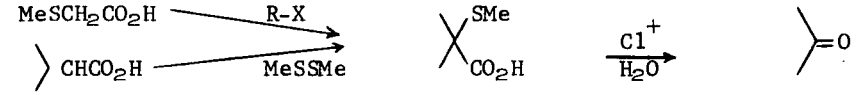
E. Other Electrophiles - Amino acids can be prepared either by direct introduction of the amino function,[65] or by elaborating hippuric acid by alkylation.[66] A nitro group can be introduced directly, and, after decarboxylation, nitroalkanes are obtained.[49] In addition to decomposition

$[PhCHCO_2]^{2-}Li_2^+ + MeONH_2 \longrightarrow PhCHCO_2H\ NH_2$ 55%

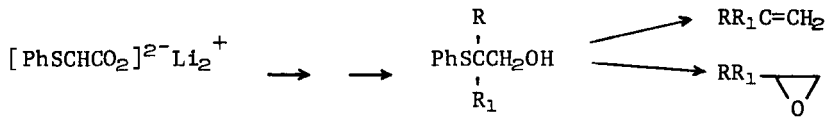
$[PhCONCHCO_2]^{3-}Li_3^+ + CH_3I \longrightarrow CH_3CHCO_2H\ NH_2$ 40%

$[C_7H_{15}CHCO_2]^{2-}Li_2^+ \xrightarrow[2.\ -CO_2]{1.\ PrONO_2} C_7H_{15}CH_2NO_2$ 68%

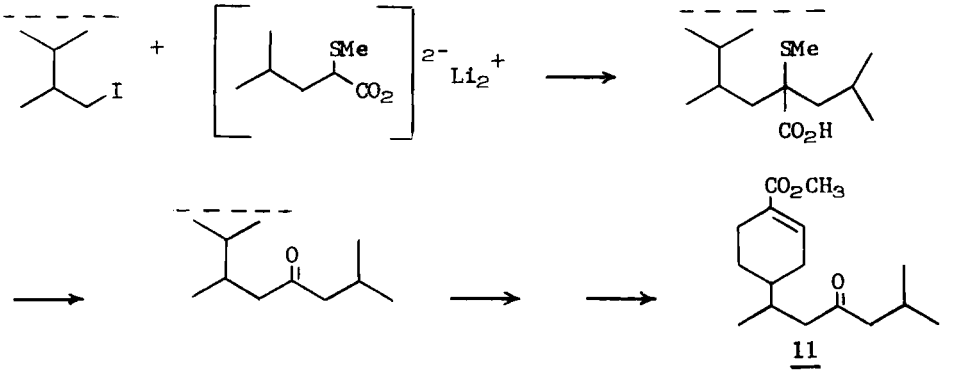
of hydroperoxides and α-hydroperoxylactones, sulfenylated carboxylic acids can be degraded to ketones.[67,68] The overall reaction provides another



example in which metalated carboxylic acids can be used as reactive carriers of the carbon residue attached to the carboxyl group; here, as an acyl anion equivalent. (Phenylthio)acetic acid has been recommended as the dianionic intermediate because of its availability.[69] The latter can be alkylated and used as a source of olefins and epoxides as well as ketones. The utility of the method has been demonstrated in the synthesis



of juvabione, 11.[68]



11

V. Conclusions - The preceding discussion should convince the reader that metalated carboxylic acids have substantial versatility in organic synthesis on both small and large scales. The literature cited suggests that much more attention has been devoted to the examination of reactions with various electrophiles than to the properties and methods of formation of the dianions upon which particular applications depend for success. It is likely that the future will provide the methodology necessary for the metalation of difficult carboxylic acids, such as, acetic and cyclopropane carboxylic acids, and insight into the structures of the

dianions as well as a better understanding of the mechanisms of reactions with different classes of electrophiles.

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Chapter 29. Computer-assisted Organic Synthetic Analysis

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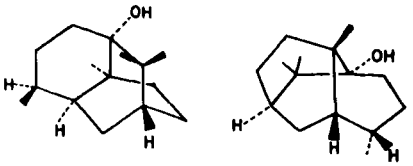
Introduction - Organic synthesis is still an experimental science. In principle, however, the application of computers to the critical planning stage of synthesis could substantially increase the synthetic chemist's efficiency.[1]

The thought processes by which the "Masters" create a successful synthesis of a complex structure remain mysterious: R. B. Woodward won the 1965 Nobel Prize in chemistry for his contributions to the "art" of organic synthesis. Corey[2] was the first to attempt a serious analysis of these processes. He identified four separate yet inter-dependent stages: choice of the molecule to be synthesized, development of an overall strategy or plan, selection and ordering of the individual steps, and experimental execution. The selection and ordering stage is most amenable to computer processing, and this aspect is relatively well developed.

The selection and ordering of steps should be done in a fairly exhaustive and unbiased, yet discriminating and systematic manner. Consider a molecule (A) that could be made from 40 different precursor compounds. If each of these precursors in turn could be made from 40 different precursors, and so on, then A could be made in five steps or less from over 100 million (40^5) potential starting materials.[3] Actually, thousands of reactions are known - although many are of unknown generality - and syntheses of more than five steps are common. The computer can untiringly attempt every reaction it "knows" at very high speeds.[3,4]

The selection and ordering of steps should be unbiased so that no obvious (in retrospect) approach is overlooked. An analysis is normally biased by the chemist's previous experience, and even by the way he draws the target molecule. For example, it is conceivable that the following two representations of patchouli alcohol could lead a chemist to propose entirely different synthetic approaches (from Ref. 5 with permission):



The computer, on the other hand, may be programmed to recognize synthetically important relationships, however the molecule is drawn.

Because of the large number of possible routes, it is necessary to discriminate between good and bad approaches. The chemist normally discards many possibilities on the basis of mechanistic organic chemical arguments, and his own experience. Similarly the computer may be programmed to evaluate steric and electronic effects, reaction energetics, interfering functionality, side reactions, and so forth, in order to discard unlikely approaches.

Ultimately, powerful synthetic strategies will restrict the generation of uninteresting routes and focus on rewarding approaches. Corey[2] has categorized some successful strategies: avoid or by-pass suspected poor yield steps, minimize correctional steps, use known reactions where possible and simplify the problem. Problem simplification may involve recognizing analogous syntheses, perceiving real or potential symmetry, recognizing important substructures (synthons), using equivalent (i.e., easily interchanged) synthons, perceiving stereochemical relationships, considering reaction energetics and kinetics, using rings or complexation to minimize functionality or provide directionality, and perceiving biogenetic possibilities.[2]

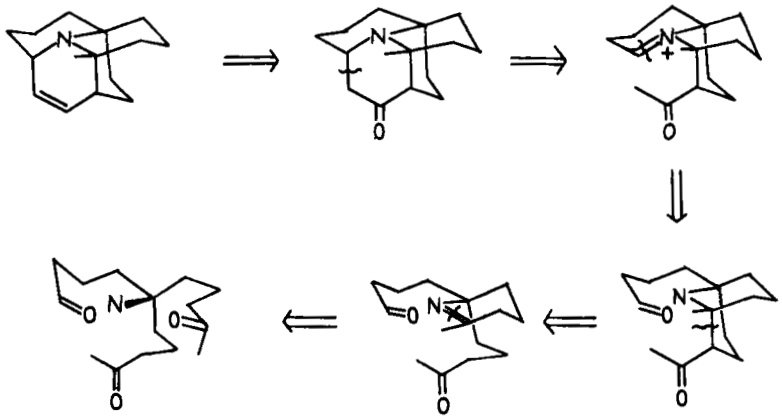
The first steps towards computerization of these strategies have been taken. For example, the concept of strategic ring bonds[6] enables an analysis to focus on breaking bonds which are likely to lead to a less complex starting material.

The final requisite for the selection and ordering of synthetic steps is that it be performed in a systematic manner. While systematization of synthesis is difficult, several attempts have been made.[2,7,8] Corey's approach[2] appears to have been most fruitful.

Logic-Centered Approach to Organic Synthesis - In this approach, formalized by Corey,[2] the chemist "works backwards", systematically generating all precursors which may be converted in one step to the product by known reactions. Each of the best precursors then becomes the target for further precursor generation, and so forth, until available starting materials are obtained. Corey listed twelve steps to be followed for this type of analysis.[2] In essence, these are to simplify the problem; recognize synthons; generate equivalent synthons; add control synthons; disconnect the synthons to create precursors; formulate the requisite reactions for these disconnections; repeat the above steps for each intermediate and each sequence; continue until a suitable starting point is reached; remove inconsistencies; identify unresolved problems; repeat the above steps to generate alternative schemes; and rate the different routes. Cyclic structures require a few additional considerations.

Not all of these steps are necessary for developing every synthetic plan, and not all types of synthetic problems are amenable to this approach.

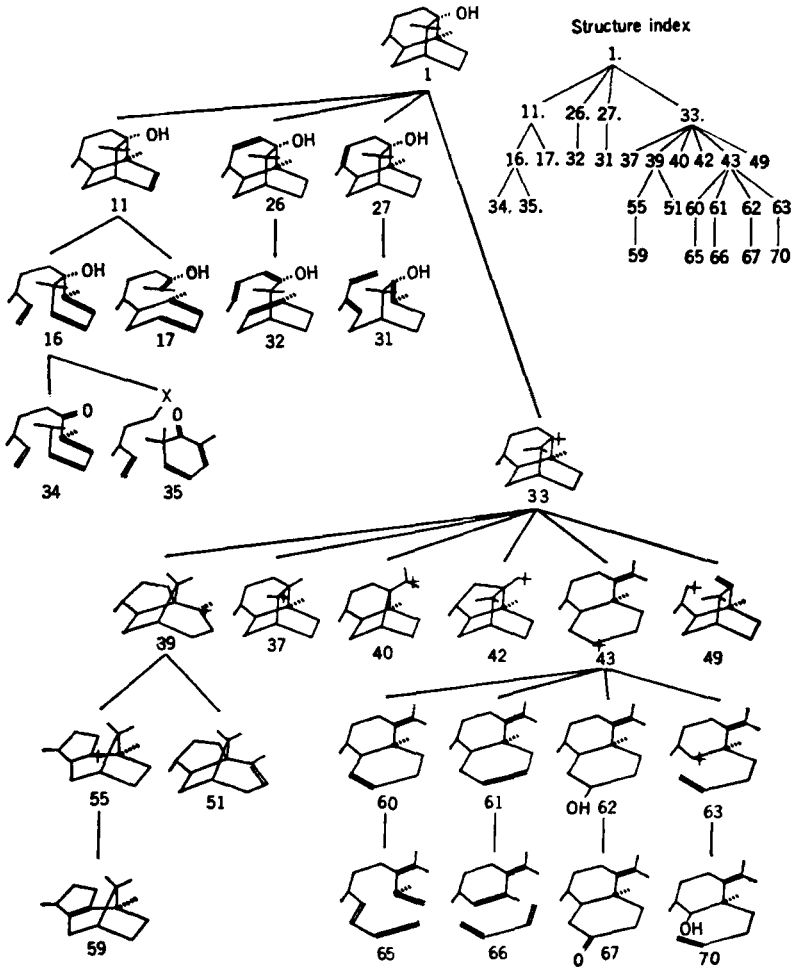
Nevertheless, where this procedure is applicable it can lead to elegant solutions to difficult synthetic problems. An example of the power of the method is found in Corey's "network analysis" of longifoline.[9] More recently,[10] a synthesis of porantherine grew out of the logic-centered analysis summarized below:



Corey noted that the twelve steps of the logic-oriented approach resembled a computer program;[2] and indeed this formalism served as the framework for the original Corey and Wipke computer synthesis program,[11] and for the later programs[3,4,12] as well.

Organic Chemical Synthesis Simulation (OCSS) - This landmark program[11] effectively removed the barrier between chemist and computer by the use of interactive computer graphics; the chemist could communicate with the machine for the first time in the language of structural diagrams.

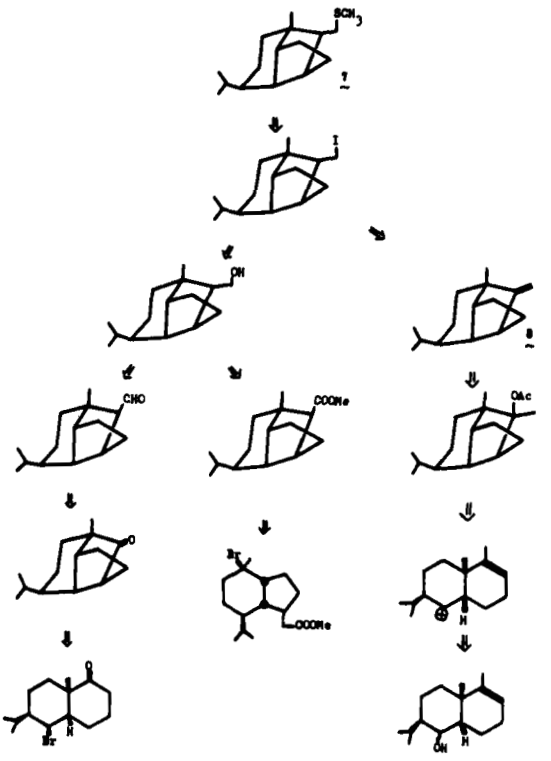
In operation, the chemist drew the target molecule on an electronic tablet, and the program generated (under chemist control) a "synthesis tree", where every node represented an intermediate structure, and the branches represented synthetic routes for obtaining the target structure. The program emulated many of the synthetic chemist's planning activities, such as molecule perception, developing a strategy, structure manipulation, and precursor evaluation. The perception module identified functional groups, rings, appendages, symmetry elements, and related properties of synthetic significance. The strategy and control section used heuristic principles ("rules of thumb" reflecting empirical knowledge about reactions) to guide precursor generation. The manipulation and control section applied symbolic "reactions-in-reverse" or "transforms" to systematically generate precursors, and the evaluation module deleted poor structures and rated the remaining ones. The quality of synthesis attainable with this early program is evident in the patchouli alcohol synthesis tree[11] (copyright 1964 by the American Association for the Advancement of Science, reproduced with permission):



Logistics and Heuristics Applied to Synthetic Analysis (LHASA) - This is a second generation program developed by the Corey group.[13] While OCSS was written in an obscure computer language (DECAL) for a now obsolete computer (PDP-1), LHASA is largely written in PDP-10 FORTRAN and is more readily adaptable to other computers.

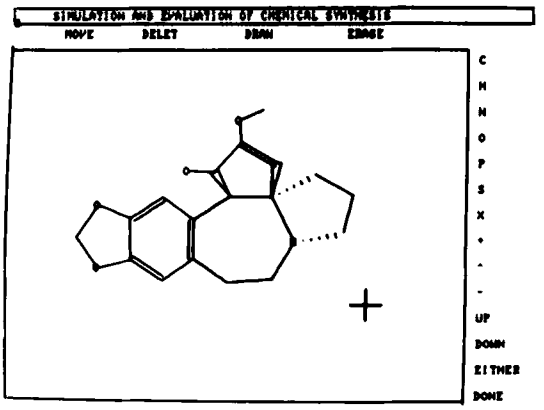
LHASA incorporates many new and expanded capabilities which can only be mentioned briefly: transforms keyed to pairs of functional groups or to a single functional group in the target molecule;[14] functional group introduction (in the retro-synthetic sense);[14] functional group interchange;[14] multistep look-ahead (i.e., accomplishing subgoal-generated transforms up to 15 levels deep in order to allow a powerful reaction to be used, before other precursors of the target are considered);[15] perception of stereochemical information;[15] very extensive and careful pro-

gramming of key ring-forming reactions, such as the Diels-Alder reaction;[15] the concept of strategic ring bonds to guide ring-forming reactions;[6] strategies keying on ring or branch appendages and using reconnective transforms;[16] sequential subgoal-generated functional group interchanges;[17] and functional group protection and deprotection.[18] A recent example[17] of LHASA-derived synthetic routes to sativene 8 and an analog 7 is shown (from Ref. 17 with permission):

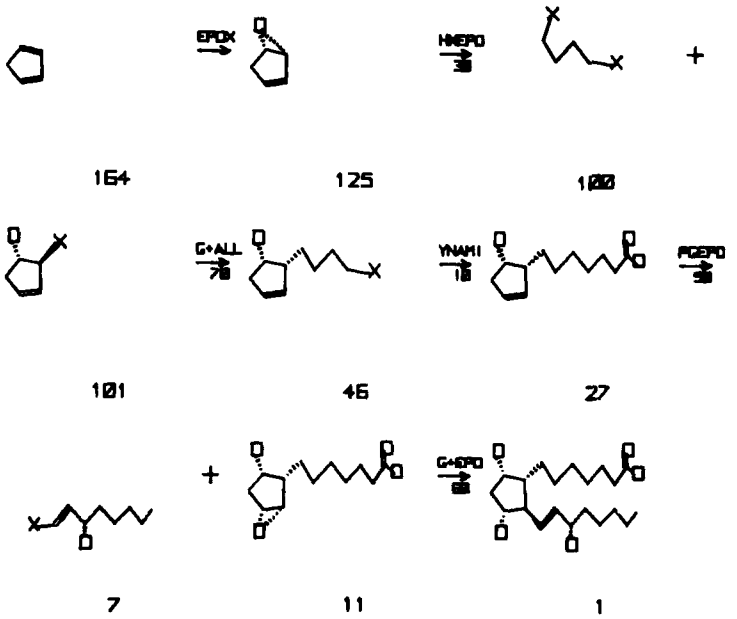


In addition to its use in synthetic research applications, LHASA has proved useful for teaching synthetic organic chemistry.[19] In an industrial environment, LHASA is being used at DuPont de Nemours & Co.[20] for exploring the generality of reactions, for planning the synthesis of pharmaceuticals and agricultural chemicals, and for discovering novel applications of synthesis to basic industrial processes. For the latter purpose, industrially important reactions are being added to the program's chemistry library.

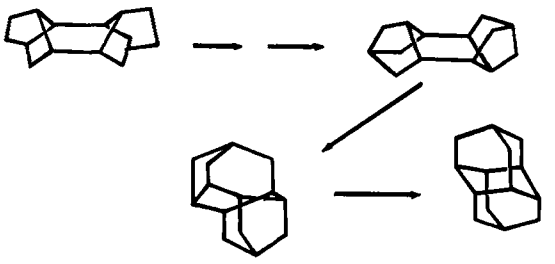
Simulation and Evaluation of Chemical Synthesis (SECS) - This program[5,12] was developed by Wipke, working independently of the Harvard group, at Princeton and more recently at the University of California at Santa Cruz. SECS is also written primarily in FORTRAN for a PDP-10 computer, with output via teletype or GT40 graphics terminal. With the latter device, the target molecule is drawn on the screen using a light pen (from Ref. 5 with publisher's permission):



SECS is currently accessible over the Stanford University SUMEX-AIM network, the Advanced Research Planning Agency network (ARPANET), and the First Data Corporation (Waltham, Mass.) commercial time-shared network. Again, the advanced features of this program can be listed only briefly: perception of stereochemical relationships;[5,21] a stereochemically unique molecule naming algorithm;[5,22] generation and recognition of enantiomers;[5,21] automatic construction of a three-dimensional model[5] and perception of steric congestion[5,23] and proximity effects therefrom; perception of aromatic and heteroaromatic directional effects from Huckel localization energies;[12] ALCHEM language for representing virtually all possible synthetic reactions in English-like sentences (reactions are keyed by pairs of functional groups; by single groups; or by very general patterns);[12] and functional group protection.[12] An example of a SECS derived route to prostaglandin $F_{1\alpha}$ is shown as displayed in the forward ("synthetic sequence") direction (from Ref. 5 with permission):



SECS proved to be useful in mechanistic as well as synthetic research. The program was used to systematically generate all Wagner-Meerwein products, with elimination of duplicate structures and calculation of strain energies, in order to find the most likely mechanism for acid-catalyzed rearrangement of tetrahydro-Binor-S to diamantane:[24]



Persistent attempts to analyze the problem by manual methods had previously failed; there are an estimated 40,000 pentacyclotetradecane isomers possible.[24]

Industry experience with SECS has been fairly widespread, with many companies evaluating the program through the First Data Corporation network. BASF, Merck/Darmstadt, Sandoz and (more recently) Bayer AG have been running SECS in Europe since 1975. SECS is also running at Merck & Co., Inc.[25]

Batch Programs - Several research groups have set themselves the task of creating batch computer programs for synthetic analysis. Since the chemist cannot intervene in these programs during execution, the program must be self-directing in further processing of intermediate structures. This requires powerful program heuristics to prevent the program from wasting time generating precursors of an uninteresting intermediate. Since it is not always obvious why certain pathways are interesting and others not, this is an artificial intelligence problem of more complexity than the well-known chess-playing program problem[26] - where the legal rules are more clearly delineated and "winning" strategies are better known.

The most advanced of the batch programs is probably Gelernter's SYNCHEM,[12,27] written in PL/I for an IBM computer. This program has the novel capability of ending when a known starting material is reached. It recognizes this condition by generating the Wiswesser line notation (WLN) name of each precursor and checking it against a computerized file of available compounds from the Aldrich catalog. SYNCHEM is being evaluated for utility in an industrial environment at Lederle Laboratories.[12] Published synthetic analyses by SYNCHEM include routes to vitamin A[27] and twistane.[27]

An assembly language program written by Bersohn[3,12] is said to be capable of generating 40,000 precursor structures per cpu minute on an IBM 370/168. Since no chemist can be expected to evaluate that many structures, Bersohn is implementing strategies to upgrade the quality of precursors generated. An earlier program[3,28] was much slower and more expensive to run.

A group headed by Ugi has aspired to implement a program which can "create" new reactions. They developed a mathematically based description of reactions in which a matrix containing all atoms and bonds of all reactants (an ensemble) was transformed into a matrix of all products and by-products.[29] A PL/I program for an IBM computer, initially named Computers in Chemistry, Logic Oriented Planning of Syntheses (CICLOPS),[30] was developed based on this approach.[12] The program's reaction library is fairly generalized, so that "creative" (unexpected) transformations frequently arise.

Other programs and formalisms have been developed, such as those by Whitlock,[31] Hendrickson,[8] Sinanoğlu,[32] and Barone,[33] but their general utility for synthesis remains to be demonstrated.

Reaction Retrieval Systems - A classical application of computers in chemistry is information retrieval, and chemical reactions are amenable to this type of treatment.[34] When a strategic plan for synthesis has been established, there is still a need for detailed consideration of reagents and reaction conditions - and a Theilheimer[7] type system may be best for this purpose. Such a file of reactions is typically searched by type of starting material, type of product, type of reaction, or conditions. Such a system usually contains very specific reactions of

unknown generality; these are generally unsuitable for the more elaborate computerized synthesis programs described above.[25] Extant reaction retrieval systems include Reactiones Organicae,[35] Derwent Chemical Reactions Documentation Service,[36] and a proprietary system developed by Imperial Chemical Industries.[12,37]

Conclusions - The synthetic chemist normally analyzes a synthetic problem until a route is found which looks like it might work; after a quick trip to the library to confirm its viability and to determine suitable reaction conditions, he goes to the laboratory. Once committed to a route, he may expend a great deal of effort to make it "go". If it fails, or if he needs to scale-up or think about patents, then the chemist will consider alternative routes. Clearly more systematic (especially computer-assisted) analysis of complex synthetic targets at the beginning may save wasted laboratory effort. Computer analysis at a later stage of the synthesis might also be useful in overcoming roadblocks.

The basic methodology appears to be well worked out. Computer perception of significant molecular features equals or exceeds the chemist's capabilities in many ways. While the computer's knowledge of chemistry is still inferior to that of a good synthetic organic chemist for most or all present-day programs, it should be kept in mind that the computer's knowledge is cumulative - and expanding rapidly.

At this stage of development, none of the computer programs appear to be particularly adept at suggesting which specific reagents and conditions are best for producing a particular product from a specific starting material. On the other hand, they often can lead the chemist to consider novel approaches to difficult synthetic problems. In the long run, the most complete synthetic analyses will arise from a partnership of chemist and computer, utilizing the different capabilities of each to the utmost.

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Chapter 30. Biochemical Procedures in Organic Synthesis

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Despite considerable documentation of their value in overcoming difficult problems in organic syntheses, biochemical procedures are still largely ignored by most organic chemists. However, the field and its enormous potential is now generating increasing interest, particularly among chemists engaged in asymmetric syntheses. Biochemically mediated reactions have not been previously reviewed in this series. Accordingly, we have elected to present a broad, and current, perspective of the subject, while including many representative examples of medicinal chemical interest.

Scope - There is an enzyme-catalyzed equivalent for almost every type of chemically catalyzed reaction.[1,2] Thousands of microbiological and enzymic transformations of chemical significance have now been documented in excellent monographs.[3] These reviews cover the field in general as well as specialized areas such as steroids, alkaloids, and terpenes. These provide access to much of the published data. Current awareness is also easily maintained.[4] So far, the use of intact microorganisms in organic syntheses has been dominant. However, wholly or partly purified[5] and immobilized enzymes[6,7] are gaining rapidly in importance.

Advantages - Compared with other catalysts used in organic syntheses, enzymes are exceptional in several respects.[8] The spectrum of reactions is very broad.[1,2] Furthermore, the generally mild reaction conditions, e.g., room temperature and neutral pH, minimize problems in sensitive molecules such as epimerization, racemization, and isomerization. However, their main advantage to organic chemists is their specificity.[8] Enzymes are usually very selective with respect to reaction type and substrate structure. Of great importance to asymmetric syntheses is their potential for discriminating enantiomers, enantiotopic[9,10] groups attached to prochiral centers, and stereoheterotopic (Re/Si)[9,10] faces of groups, e.g., C=O and C=C.[8-10] For many enzymes,[5,11] and some microorganisms,[12] the stereochemistries and specificities of the catalyses are well established, and structures and absolute configurations of products can be projected with confidence. Such knowledge enables preselection of the best enzyme to ensure formation of only the active stereoisomer of a drug, or to effect stereospecific introduction of an isotope prior to metabolism studies.

Factors to be considered - Biochemical methods of effecting chemical transformations are often expensive. However, their high "pay-off" can often more than offset this factor, particularly when the target molecules are costly to produce as is the case with most drugs. For example, using chemical reagents, tedious multistep procedures are required to effect controlled oxygenation at unactivated carbon, or to achieve selective oxidation of only one hydroxyl group in a poly-OH molecule, especially if

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transformation of only one enantiomer of a racemate is desired. Such reactions are readily effected enzymically in a single step. For chemists, the most useful enzymes are those which tolerate broad structural variations but retain high stereospecificity. Currently, mammalian enzymes fit these requirements best;[8] microorganisms generally have rather narrow structural specificities,[12] and it is often necessary to screen a number of organisms to find the one best suited to the substrate involved. The levels of the desired enzyme in many instances, can then be increased by induction.[12] Several factors can have adverse effects on enzymes and microorganisms. These include elevated temperatures, pH changes, added salts, organic solvents, and inhibitors. Information on these aspects is available from several sources.[3,5,13-15]

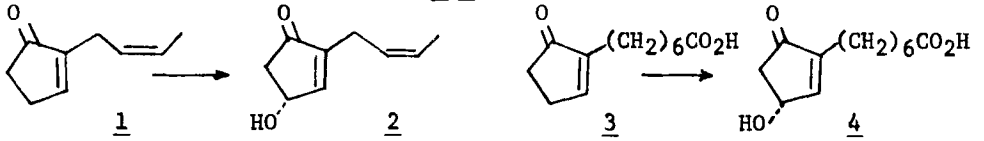
Coenzymes - Many enzymes require nonprotein coenzymes for catalytic activity.[8] These are cosubstrates, and must be constantly reconverted into their active form for catalysis to continue. This is not a problem for growing microorganisms since the normal metabolic processes ensure an adequate supply of coenzymes. However, with purified, or immobilized enzymes, maintaining a sufficient concentration of coenzyme can pose a major problem. Coenzymes are expensive[16] and it is seldom economically feasible to add them in stoichiometric amounts. This is often undesirable for chemical reasons, e.g., the coenzyme may be unstable, or the eventual build-up of high concentrations of its inactive form may induce displacement of an equilibrium reaction in the opposite direction to that desired.[5] It is therefore necessary to use catalytic amounts of coenzymes and to ensure that the active forms are continuously regenerated. Some coenzymes present little or no problem in this regard since they are automatically reformed under the normal aqueous reaction conditions or in the presence of oxygen. These include biotin, pyridoxal phosphate (PLP), thiamine pyrophosphate, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).[16] Others, such as adenosine triphosphate (ATP), coenzyme A, folic acid, nicotinamide adenine diphosphate [NAD(P)/H], $FMNH_2$ and $FADH_2$, must be continuously reformed from their inactive precursors by incorporating an auxiliary, usually in situ, regenerating system into the reaction mixture.[5,16] Recycling of the nicotinamide coenzymes and of ATP have received the most attention so far. The development of economical ATP-recycling systems now seems assured.[16,17] In contrast, despite the considerable effort that has been expended,[5,16-22] no generally applicable and industrially viable NAD(P)/H recycling systems have been reported. While recycling efficiencies of 2×10^4 are attainable for brief periods under idealized conditions,[17] 10-50 fold turnovers are more normal for preparative-scale oxidoreductions.[5] Nevertheless, the available recycling methods, which have been recently reviewed comprehensively and critically,[5] are more than adequate for research scale applications of NAD(P)-dependent alcohol dehydrogenases.[5,23-25]

Experimental Procedures - A good knowledge of the principles and techniques of both organic chemistry and biochemistry is highly desirable, but an extensive background in microbiology is not necessary. With purified enzymes, the methods used are essentially those of organic chemistry.[5] Carrying out fermentations and working with immobilized enzymes and microorganisms requires that a chemist develop some new skills, which are easily acquired.

Straightforward illustrative procedures, often written with the chemist in mind, are available.[3,7]

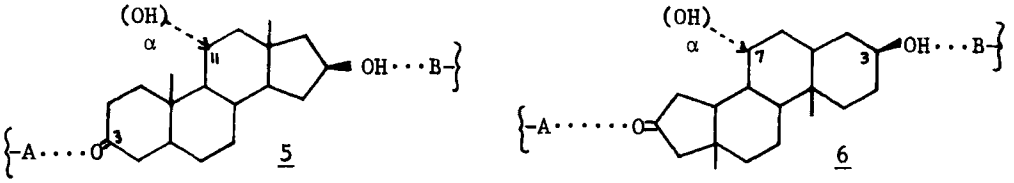
Some Recent Examples - Since organic chemists have become accustomed to predicting the consequences of using chemical reagents, only biochemical transformations for which the various aspects of specificity can be accurately forecast were considered when the examples cited below were selected.

Oxidoreductases - Controlled and stereospecific hydroxylation at unactivated carbon is easily achieved using fungi.[12] The chance of finding an appropriate microorganism for a given hydroxylation is improved if a suitable literature analogy can be found. For example, the conversion of 1→2[26] proved to be a good model for the 3→4[27] (prostaglandin synthon).



1 2 3 4

A rational basis for interpreting the regiospecificity of steroid hydroxylation is emerging.[12,28,29] With Calonectria decora and Rhizopus nigricans, the orientation of a steroid substrate appears to be controlled by interaction of the oxygen functions with hydrophilic sites (such as A or B) at the active sites of the hydroxylases. Thus 5 and 6 bind in a similar manner and hydroxylation occurs from the same active site location to give the 11α- or 7α-OH product respectively,[28] approximately 5.5 Å away from the initial oxygen function. This relationship was also observed in hydroxylations of cyclic alcohols and acylated amines by Sporotrichum sulfurescens.[30]



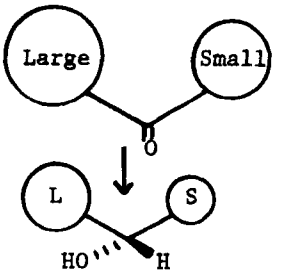
5 6

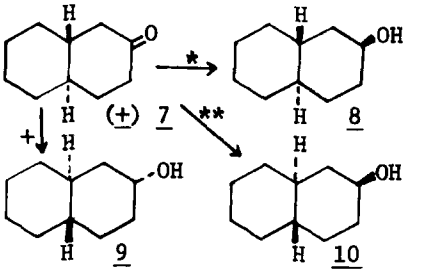
Immobilization does not destroy hydroxylase activity. 11β-Hydroxylation of 17α,21-dihydroxy-pregn-4-ene-3,20-dione by Culvularia falcata supported in polyacrylamide has been reported.[31] This flow procedure was coupled with a polyacrylamide-immobilized $\Delta^{1,2}$-dehydrogenase of Arthrobacter simplex so that the cortisol produced was converted directly into the more active corticoid, prednisolone. The $\Delta^{1,2}$-dehydrogenase of A. simplex has also been employed for resolving synthetic steroids and for determining their absolute configurations.[32]

Hydroxylations of aromatic substrates follow the rules of electrophilic substitution.[12] L-Dihydroxyphenylalanine (L-DOPA),[33] and hydroxylated metabolites acronyane,[34] 5-anilino-1,2,3,4-thiatriazoles[35] and α-methylfluoren-2-acetic acid[36] have been prepared in this way.

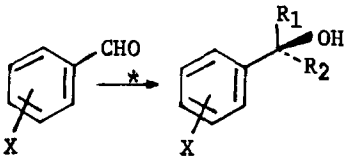
Alcohol Dehydrogenases - These enzymes catalyze C=O⇆CH(OH) oxidoreductions.

With few exceptions, e.g., Mucor javanicus,[5,37] their stereospecificities obey the Prelog rule.[5,12] This has been exploited in several prostaglandin preparations.[12] Considerable control can be exerted over the stereochemistry during oxidoreduction as is demonstrated by the formation of 8, 9, and 10 from (+) 7.[5] For these and other enzymes sophisticated "diamond lattice" active site models are available for making stereochemical predictions.[5]

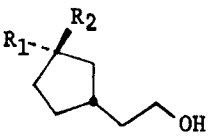




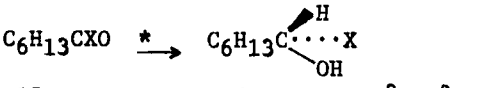
Alcohol dehydrogenases are also ideally suited to stereospecific introduction of ^{2}H and ^{3}H. Elegant enzyme catalyzed exchange processes provide facile routes to the R and S enantiomers of alcohols RCH[^{2}H]OH and of [2-^{3}H]-lactate, -malate and -glutamate.[24] Biosynthetically-useful ^{3}H-alcohol enantiomers such as 11 and 12 are also easily prepared.[23] An analogous enzymic reduction is the key step in the recent preparation of (5S)-[5-^{3}H]-mevalonic acid.[38] Oxidation of (+) 13 to 14 occurs with high regio and enantiomeric specificities.[25] Many alcohol dehydrogenases also exhibit enantiotopic specificity of practical value.[5] In the reduction of 15 to 16,[39] and 17 to the steroid intermediate 18,[40] and oxidation of 19 to 20,[25] the oxidoreduction is stereospecific for pro-S C=O and OH groups, respectively.



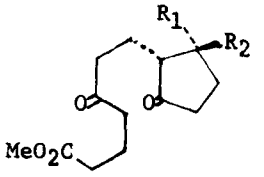
11: R_1 = H, R_2 = ^{3}H
12: R_1 = ^{3}H, R_2 = H



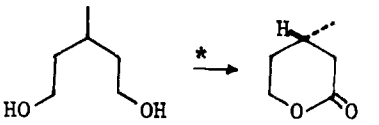
(+) 13: R_1 = OH, R_2 = H
*14: R_1 = R_2 = O



15 16 X = ^{2}H, ^{3}H



(+) 17: R_1 = R_2 = O



19 20

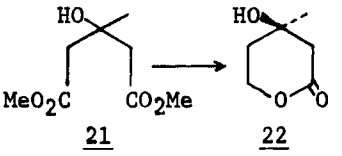
(+) 18++: R_1 = H, R_2 = OH

*Horse liver alcohol dehydrogenase (HLAD) **M. javanicus
+C. falcata ++R. arrhizus

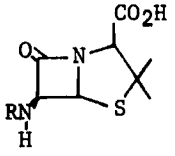
Hydrolases - Chymotrypsin is the best documented hydrolase. It has a very broad and predictable stereospecificity and is widely used in resolution of racemic esters.[1] L-DOPA has been prepared in this way.[41] Several esterases also exhibit enantiotopic specificity.[1] This is exploited in the synthesis of R-mevalonolactone 22 from 21.[42]

Large scale resolution of amino acids is an area where immobilized enzyme technology has triumphed. L-Phe and L-Met are now continuously produced on a 20 ton/month scale via immobilized aminocyclase hydrolysis of their racemic N-acetyl precursors.[43] Similarly L-Ly has been obtained by yeast hydrolysis of (±)-α-amino-ε-caprolactam.[44] The value of mild and specific enzyme procedures when dealing with sensitive molecules is demonstrated by the conversion of 23 to 24.[45]

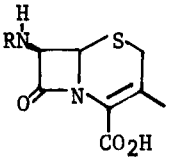
Operation of hydrolases in the reverse direction to effect acylation is also viable, as shown by the preparation of 26 from 25.[46] Hog kidney acylase has been used to resolve (2RS,3RS)-valine-$[^2H]_3$ for cephalosporin biosynthesis.[47]



21 22

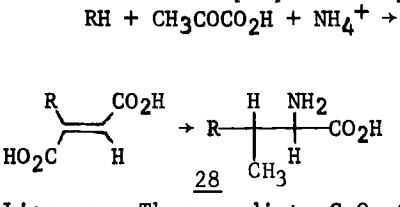


23: R = $PhCH_2CO$
24: R = H



25: R = H
26: R = PhCHCO (NH2)

Lyases - These are enzymes that catalyze addition of HX to C=C, C=O, and C=N bonds. The stereochemistry of addition is established as anti for all 17 C=C additions studied to date.[11] This is again an area where immobilized enzyme methods are superior. Conversion of fumaric acid to L-aspartic[48] and L-malic[49] acids are two such processes with commercial potential. The most impressive example of lyases in asymmetric syntheses is the synthesis of L-amino acids, 27, using immobilized β-tyrosinase or tryptophanase.[50] Oxynitrilase-catalyzed additions of HCN to aldehydes to give cyanohydrins can be carried out on the kilogram scale. The D-cyanohydrin products are readily converted to other useful optically active synthetic starting materials, e.g., hydroxy acids, hydroxy amines, and acyloins.[51] The labelled valines 28 used in cephalosporin and penicillin biosynthetic studies are best prepared enzymatically.[52]



28

$RH + CH_3COCO_2H + NH_4^+ \rightarrow RCH_2CHCO_2^{\ominus}$ (L-27), $\oplus NH_3$

R = 4-hydroxyphenyl, 3,4-dihydroxyphenyl, 3-indolyl, 3-(5-hydroxy)indolyl

R = $^{13}CH_3$, C^3H_3

Ligases - These mediate C-O, C-S, C-N, and C-C bond formation. Phosphate bond-forming enzymes have been used most widely. Coenzyme A is conveniently made, using a polyacrylamide-supported synthetase to couple ATP and pantethine.[53] The use of phosphorylases has also solved some severe

(poly)nucleotide coupling problems of nucleic acid syntheses. Examples include syntheses of the termination codons UAA, UAG and UGA.[54] Moreover, large (20-30 g) amounts of the poly I-C interferon inducer[55] have been prepared using immobilized enzyme techniques. The elegant uses of a T-4 ligase to join polynucleotide phosphate fragments of the alanine t-RNA of yeast and tyrosine suppressor t-RNA of E. coli genes represented key steps in the respective syntheses of these two giant molecules.[56]

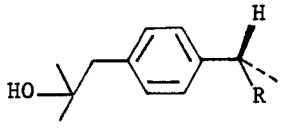
Having surveyed the applications of biochemical systems to organic syntheses, it may be appropriate to follow this up with a discussion of the strategies used in some combined classical-biochemical syntheses. The application of microbes or enzymes for the generation of chiral centers in asymmetric syntheses constitutes one of the most valuable tools available to the synthetic chemist. Although achievement of the desired transformation is heavily dependent upon the judicious selection of microbes or enzymes that possess broad substrate specificities while catalyzing reactions with high degree of stereospecificities, the chemist plays a significant role in designing substrate molecules suitable for biochemical asymmetric inductions. We herein illustrate some approaches to the development of substrate molecules with prochiral centers in some combined asymmetric syntheses. Hopefully, this rationale may also be applicable to other problems.

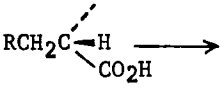
The recent introduction of arylpropionic acids as anti-inflammatory drugs[57] has aroused considerable interest in improved methods for their preparation. It is noteworthy that with some arylpropionic acids such as p-isobutylhydratropic acid[57] (29) (Motrin), the individual S(+) and R(-) isomers were essentially biologically equivalent. This equivalence has been shown to be due to the in vivo conversion of the R(-) to the S(+) isomer with labelling studies. R(-)-p-isobutylhydratropic acid[58] was converted to the S(+) isomer with the loss of two deuteriums suggesting the following pathway of epimerization.

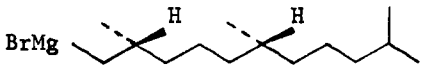
p-i-$C_4H_9C_6H_4CH(CH_3)CO_2H$ 29

$$R(-) \rightarrow Ar\overset{CD_3}{\underset{|}{C}}HCOSCoA \rightarrow Ar\overset{CD_2}{\underset{|}{C}}HCOSCoA \rightleftneq Ar\overset{CD_2}{\underset{||}{C}}COSX \rightarrow Ar\overset{CD_2H}{\underset{|}{C}}HCOSCoA \rightarrow S(+)$$

For other anti-inflammation drugs such as Naproxen[59] and Fenoprofen,[60] only the S-isomer is biologically active. Since tedious resolutions are necessary to prepare these compounds, it would be desirable to develop a general asymmetric synthesis of the S(+) arylpropionic acids by microbial oxidation of pro-chiral substrates. This concept has been successfully applied in the preparation of a chiral metabolite of 29. Oxidation of one of the enantiotopic methyl groups in 30 with S. sulfurescens furnished the chiral diol 31.[61] A similar oxidation of isobutyric acid (32)[62] to S-(+)-3-hydroxy-2-methylpropionic acid (33) has been recently employed in the synthesis of the α-tocopherol synthon (34).[63]



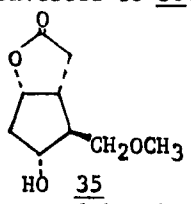




30: R = CH_3 32: R = H 34

31: R = CH_2OH 33: R = OH

One of the key intermediates in Corey's total synthesis of prostaglandins[64] is the lactone (35) whose optically active form was obtained by resolution of (+) 36. Upon analysis of the sequence of reactions from cyclopentadiene to 35 one would readily visualize the possibility of carrying out microbial resolution of various intermediates along the synthetic pathway. One such resolution has been accomplished by microbial reduction of the (+) ketone 37 with Saccharomyces drosophylarum to yield the (+) exo and (-) endo alcohols (38 and 39). These were separated by chromatography and were oxidized to the optically active ketones (37).[65] One of these was converted to 36.[64]



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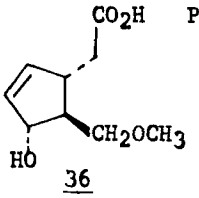

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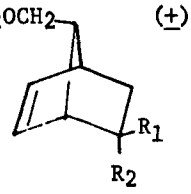
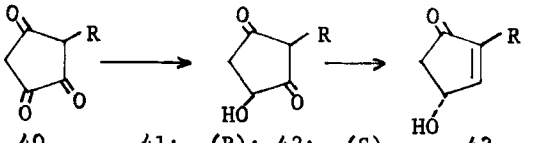


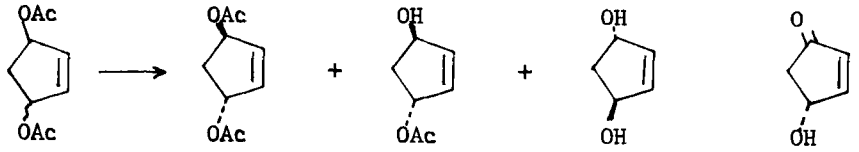
(+) 37: $R_1 = R_2 = O$
38: $R_1 = H$, $R_2 = OH$
39: $R_1 = OH$, $R_2 = H$

Although these microbial reactions may substitute for chemical resolution methods, they likewise have the inherent disadvantage that only one half of the material is theoretically utilizable, for the substrate is already chiral. It is therefore preferable to design substrates with prochiral centers suitable for microbial asymmetric inductions. Examples that have been applied in the prostaglandin area follow. Reduction of triketone 40 with Dipodascus uninucleatus and Mucor rommanianus furnished the (R) and (S) alcohols (41 and 42) respectively.[66] The (R) alcohol (41) was then chemically converted into 43, a key synthon for prostaglandin synthesis via conjugate addition.



40 41: (R); 42: (S) 43 R = $(CH_2)_6CO_2Me$

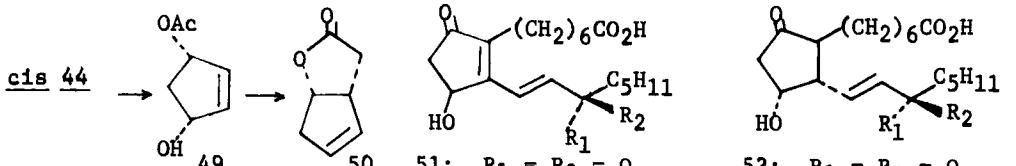
Since R-48 is also an important versatile synthon for prostaglandin synthesis, there has been interest in devising asymmetric methods for its preparation. Japanese workers[67] subjected a 1:1 mixture of cis and trans 44 to esterases from baker's yeast and were able to obtain the optically active (R,R)-45, (R,R)-46 and (S)-predominant 47. Thus a simultaneous kinetic resolution of the diacetate (44) and asymmetric synthesis of the monoacetate (46) were effected by this hydrolysis. These were converted to prostaglandin synthons.[68]



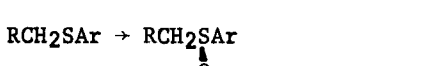
44 45 46 47 48

In a related work hydrolysis of the pro-chiral cis-44 using Bascillus subtilis var Niger furnished the chiral alcohol (49).[69] This was in turn converted to the lactone 50, another prostanoid synthon.[70]

Although stereoselective chemical reduction of the C-15 carbonyl function in prostaglandins has been achieved,[71] it would be desirable to find a microbe capable of catalyzing the reduction of the C-15 carbonyl group stereospecifically yielding the desired (S)-alcohol. Partial success in this area has been achieved when (+) 51 was reduced biochemically where Flavobacterium sp. NRRL B-3874 and Pseudomonas sp. NRRL B-3875 produced stereoselectively the (-) and (+) forms of 52, respectively.[72] While these transformations proceeded stereospecifically, these enzyme systems require α,β,δ,γ-unsaturated diketones for reduction. Although it is generally accepted that α,β-unsaturated ketones are not reduced to allylic alcohols biochemically,[73] Trechiopora brinkmanii has been found to reduce 15-keto-prostaglandins in the E and F series to the corresponding 15(S) alcohols illustrated by the conversion of 53 to 54.[74]



The chemical method of choice for the preparation of chiral sulfoxides is complicated by the formation of undesirable side products.[75] It entails the addition of a Grignard reagent to ℓ-menthyl-ℓ-arenesulfinates[76] producing one enantiomer whose antipode has to be obtained by chemical inversion.[77] Microbial oxidation of thioethers is, therefore, an attractive method to prepare these chiral reagents. A variety of sulfides have been oxidized by a number of fungi, e.g., Aspergillus niger, occasionally producing sulfoxides of high optical purity.[78] Methyl tolyl 55 as well as benzyl phenyl sulfide (56) have been oxidized to the corresponding R-sulfoxides (57 and 58) in high optical purity. It is clear that a concerted effort in this area is needed to find organisms that produce both R and S sulfoxides of synthetic utility.



55
56

57: R = H, Ar = p-$CH_3C_6H_4$
58: R = Ar = C_6H_5

Conclusion - The application of biochemical systems to the solution of organochemical problems will continue to expand rapidly, and the few representative examples described herein constitute only the beginning of a very dynamic field. The successful utilization of biochemical chiral reagents is heavily dependent upon the design of suitable substrates, an area where the synthetic chemists can play a major role. It is hoped that before too long chemists will consider biochemical methods a routine powerful addition to their arsenal of synthetic reagents.

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Chapter 31. Organic Electrosynthesis

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The fundamental question addressed here is, "Can useful or unique reactions be performed in high yield, on a synthetic scale, using electrochemistry?" The answer is "yes".

Organic electrosynthesis is not a new technique and there is considerable knowledge of the types of reactions that take place at cathodes and anodes. It is also true, however, that there have been few attempts to develop electrochemical reactions as general synthetic tools and very few attempts to use electrochemistry to synthesize complex molecules. Therefore, electrosynthesis is at present a promising, but seldom tested technique for the synthesis of medicinals.

Our view of organic electrochemistry is that it is a unique, nonthermal method for activating molecules. Since the rate of reaction can normally be increased by raising the electrode potential, it is possible to carry out reactions with a high activation energy at low temperatures. Indeed, very highly energetic intermediates can be produced. For example acetonitrile containing a fluoborate electrolyte oxidizes only at potentials above 3.2 V vs a saturated calomel electrode (SCE) reference. This means that oxidations with an activation energy of 74 kcal/mole more endothermic than SCE can be performed without oxidizing the solvent-electrolyte. In this range one can directly oxidize most organic compounds including aliphatic hydrocarbons.

Another view is that electrochemistry is an alternative to chemical redox methods. Indeed, in certain cases the products are similar. This is to be expected if the chemical reagent reacts like an electrode via discrete electron transfer steps - not atom transfers. Even here, however, it is not unusual to observe significant differences between chemical and electrochemical processes. A peculiar advantage of electrochemistry is control of the electrode potential. In particular one can adjust the potential to selectively attack the most easily reduced or oxidized moiety in a complex molecule. This technique can also avoid the over-reductions and oxidations produced by chemicals.

These views then characterize an electrode as a powerful reagent which, through potential control, has considerable selectivity. An important further consideration is that large amounts of material can be produced. This has been most dramatically demonstrated by the commercial production of adiponitrile.

It should be understood that it is not necessary to have an intimate knowledge of the Ilkovich equation or the vagaries of double potential step chronoamperometry to do preparative electrochemistry. Furthermore, it is technically simple. The equipment can be as simple as a DC

power supply, beaker and electrodes. A divided cell is, however, often important and access to a potentiostat is desirable. The advantage of a cell separated into anode and cathode compartments by a glass frit or membrane is illustrated by the reduction of a ketone to an alcohol. Ketone is added only to the cathode compartment and the product alcohol is retained there. This keeps the alcohol from being oxidized at the anode and keeps unwanted anodic products out from the catholyte. As noted above one of the advantages of electrochemistry is the possibility of controlling the electrode potential. This can be accomplished most simply through the use of a potentiostat. Models most useful for synthesis are available for about $3,000. The use of such a machine can be mastered by anyone who can operate a radio and there is little problem with maintenance. Once the proper potential has been established in a small scale (a few grams) experiment, it is not necessary or desirable to use a potentiostat for producing large amounts of material.

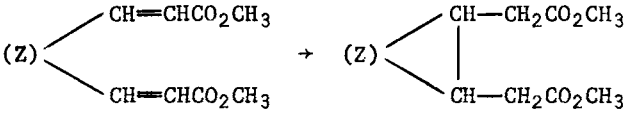
More information on what can be done and how to do it can be found in a number of books which have appeared in the past few years. Thorough coverage of the literature up to about 1972 can be found in the volumes edited by Baizer[1] or by Weinberg.[2] Useful introductions for medicinal chemists are in Eberson and Schaefer's review[3] and in Fry's book.[4] In this brief review we enucleate a few reactions which have been proven to work for complex molecules or which have demonstrated utility for the preparation of simpler organic compounds. No attempt has been made to be comprehensive and only a few leading references are included. Our primary goal is to increase the reader's awareness of electrosynthesis.

Carbon-Carbon Bond Formation - A number of anodic and cathodic coupling reactions are known. The Kolbe oxidation of carboxylate salts and pinacol formation from ketone reduction are familiar examples. Somewhat less well known is the reductive coupling of activated alkenes.

$$R{-}CH{=}CH{-}X + 2e^- + 2H^+ \rightarrow XCH_2\underset{R}{C}H\underset{R}{C}HCH_2X$$

X = CN, CO_2R, COR

This hydrodimerization process has been very thoroughly studied by Baizer and coworkers and others.[5] A number of examples have been performed and the yields are usually >90%. Because variations in yield with many conditions, e.g., solvent, cathode material, have been studied and the mechanism has been elucidated, there are many possibilities for performing this reaction in high yield for other substrates. One of the most interesting applications is intramolecular coupling.[6]



Z = $-(CH_2)_n-$, $-(CR_2)-$, $-(OCH_2CH_2O)-$

Mixed coupling has also been achieved in certain cases.[7] High yields of mixed dimers can be obtained by selectively reducing (controlled potential) one alkene in the presence of a second which is more reactive as a Michael acceptor. In the following example the reduction is performed at -1.75 V

and the mixed dimer is formed in high yield.

$$CH_2{=}CHNHCOCH_3 + CH_2{=}CHCN \rightarrow NCCH_2CH_2CH_2CHNHCOCH_3$$
$$\quad\quad CO_2CH_3 \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad CO_2CH_3$$
$$E_{1/2} = -1.7 \qquad\qquad E_{1/2} = -1.9$$

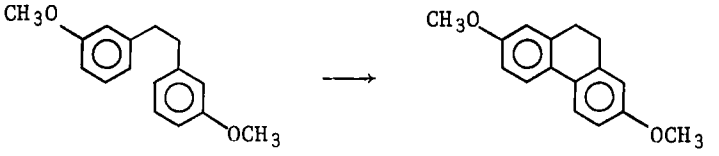
If the cathode potential used is more negative, more of the symmetrical dimers are obtained in competition. Typical conditions for hydrodimerization involve the use of aqueous solutions containing tetraalkylammonium tosylates (with a cosolvent, if necessary) and alkene. Mercury and lead cathodes give highest yields.

The mechanism of hydrodimerization has been elucidated[8] and illustrates some general considerations.

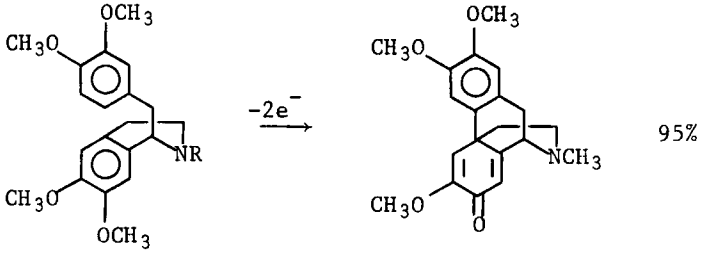
$$e^- + CH_2{=}CHCN \rightarrow CH_2{=}CHCN^{\bullet -}$$
$$2CH_2{=}CHCN^{\bullet -} \rightarrow NC\overline{C}HCH_2CH_2\overline{C}HCN \xrightarrow{2H^+} NH(CH_2)_4CN$$

The initial intermediate, as in most reductions, is the anion radical. Its dimerization produces only the most stable 1,4-dianion which is finally protonated. The kinetic acidity near the surface is quite important because at high pH the anion radical will be diverted by protonation before coupling and in aprotic media polymerization results.

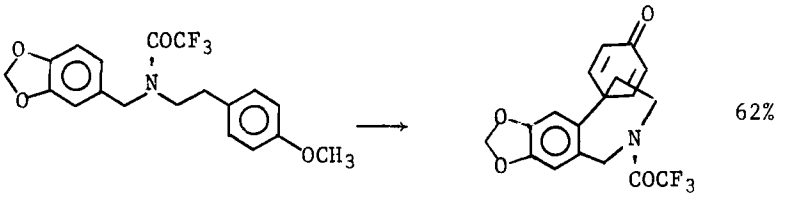
Anodic coupling of vinyl ethers, phenols and phenol ethers shows considerable promise for the synthesis of natural products and medicinals. Both inter- and intramolecular coupling is possible. Interest has centered on the cyclization of bibenzyls. A simple example is found in the work of Parker and Ronlan.[9]



Applications to alkaloid synthesis are exemplified by the construction of the morphinan ring system[10](I) and synthesis of the oxocrinine analog (II).



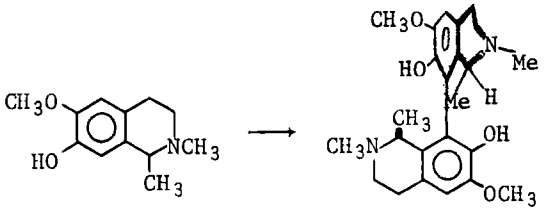
I



II

In each of these biomimetic examples cyclization occurs preferentially para to an alkoxy leading to cyclohexadienone products. Oxidation of the corresponding phenols using chemical or electrochemical methods gives much lower yields.

Bobbitt and coworkers have reported a number of intermolecular phenol coupling reactions, the most intriguing being a stereospecific phenol coupling reaction.[12] Using a carbon anode they were able to produce only



one of the three possible diastereomeric dimers. This is clearly attributable to reaction at the anode surface. It illustrates that because of the heterogeneous and catalytic nature of an electrode reaction, one has some unique possibilities for selectivity.

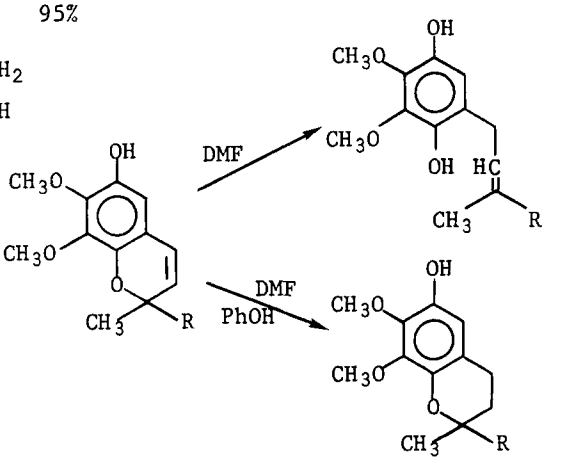
Very recently it has been shown that electrode surfaces can be chemically modified.[13] Although no useful reactions have come from this work, it has been shown that organic molecules can be covalently attached to electrode surfaces and that these modified surfaces impart selectivity to electrochemical reactions which is not otherwise available. Attempts have also been made to increase the selectivity of electrochemical reactions by adsorbing material on the electrode surface. In particular if chiral alkaloids are adsorbed on mercury, it is then possible to perform the asymmetric reduction of prochiral ketones to chiral alcohols. An optical yield of 54% has, for example, been reported for the reduction of 4-acetyl pyridine in aqueous-ethanol using strychnine as the catalytic, chiral reagent.[14]

Cleavage Reactions - Two of the most common cathodic processes are hydrogenation of double bonds (C=C, C=O, C=N, N=N) and cleavage of C—X bonds.[15] Virtually any organic bromide or iodide is electroactive and can be cleaved under the proper conditions. The products from RX are generally RH and/or RR depending on R and the conditions. Cleavage of Cl, OH, NR_2, OR, and CN is also possible, but in these cases it is generally necessary to have an activating group in the molecule. The electrochemistry of

functionalities like $Ar—CH_2X$, ArX, $-C{\equiv}C-CH_2-X$, $-COCH_2X$, $-CHX_2$ have, for example, received study. We show here a few cases of cathodic C—O bond cleavage.[16-18]

$$Ph_2C{=}CH-CH_2OH \rightarrow Ph_2C{=}CH-CH_3 \qquad 95\%$$

$$HOCH_2-CO-\underset{OH}{\underset{|}{CH}}-\underset{OH}{\underset{|}{CH_2}} \rightarrow CH_3-CO-\underset{OH}{\underset{|}{CH}}-\underset{OH}{\underset{|}{CH_2}}$$



$$R = CH_2CH_2CH{=}\overset{CH_3}{\overset{|}{C}}-CH_2CH_2CH{=}\overset{CH_3}{\overset{|}{C}}CH_3$$

The latter reactions demonstrate the general phenomenon that aprotic solvents favor cleavage and protic media favor hydrogenation. Both pathways involve direct electron transfer from the cathode to the substrate. The resulting anion radical can either cleave the C—O bond or be protonated on carbon by phenol. The former reaction leads to cleaved hydrocarbon by a second electron transfer and protonation. The latter process is also completed by electron and proton transfer giving the saturated alcohol.

A major use of reductive cleavage is deprotection. This has been investigated for the case of alcohols and amines which have been protected as tosylates.[18] Using DMF and a Hg pool cathode, 70-80% yields of the corresponding alcohols or amines are obtained from simple tosylates or tosylamides. Application to amino acid synthesis is successful[19] and although this method would presumably have limitations in polypeptide synthesis, it is useful for mild, economical, large scale deprotection.

$$\underset{\underset{NH—Tos}{|}}{RCH—CO_2^-} \rightarrow \underset{\underset{+NH_3}{|}}{RCH—CO_2^-}$$

Yields of 65-90% are obtained on a 50 g scale for fourteen different amino acids. Cleavage of benzyl and carbobenzoxy groups[18] is also possible.

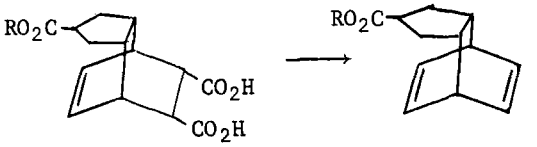
These deprotection reactions exist as alternatives to catalytic hydrogenation and acid catalyzed hydrolysis. Another alternative for alcohol deprotection is anodic oxidation.[20]

$$Ar-CH_2-O-R \xrightarrow{-2e^-} ArCHO + ROH$$

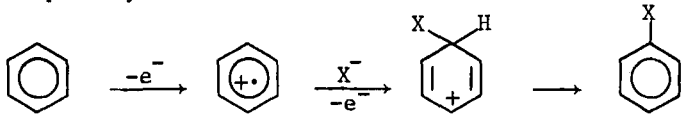
The simple alcohols are released in good yield and provide a complimentary technique which avoids reduction or hydrolysis of other sensitive portions of a complex molecule.

Another anodic cleavage reaction which has been used is bis-decar-

boxylation.[21] Its utility derives from use of maleic anhydride as a dienophile in the construction of polycyclic aliphatics. An example is:



Anodic Substitution - In the past few years a number of anodic substitution processes have been discovered.[22] The scope of these reactions is defined and often they provide the method of choice for performing the particular transformation. These processes generally use non-aqueous solvents which have a combination of polarity and relative inertness to oxidation. A general scheme is: RH + Nuc → RNuc + H^+. Both aromatic and aliphatic compounds have been substituted in this way and C—O, C—N and C—C bonds can be formed. Considering aromatic substitution, the mechanism generally involves direct electron transfer from the aromatic to the electrode. Although the timing of nucleophile attack and loss of a second electron has not been elucidated and the importance of the surface is generally unknown, a conceptually useful mechanism is:



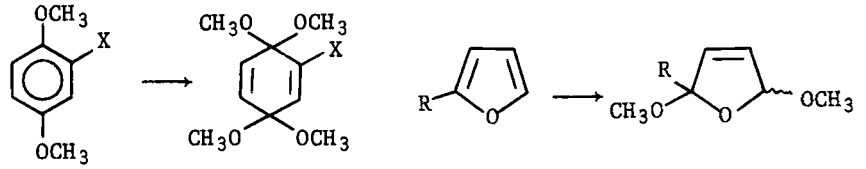
Ar-$NHCOCH_3$, Ar-O_2CR and ArCN have been synthesized in this way[22] and the position of substitution can often be predicted from calculation of charge densities in the cation radical.

For Ar-O bond formation, the solvent is often used as the nucleophile. Thus, for example, a mixture of trifluoroacetic acid and nitromethane is sufficiently conductive, does not oxidize at potentials up to 2.4 V vs Ag/Ag^+ and traps cations formed in oxidative processes to form trifluoroacetate esters. Using this medium for electrolysis and an aqueous work-up, it is possible to perform aromatic hydroxylation reactions, often in yields of 75%.[23]

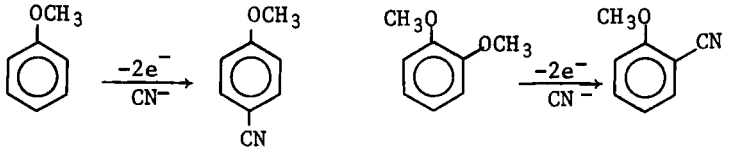
$$ArH \xrightarrow{-2e^-} ArO_2CCF_3 \xrightarrow{H_2O} ArOH$$

Aromatic hydroxylations of any kind tend to be unsuccessful because the phenol produced is more reactive than the reactant, ArH. In this case, however, only the trifluoroacetate ester, ArO_2CCF_3, is present in the electrolysis mixture, and it is less reactive than the original ArH. One of the more interesting aspects of this reaction is that aromatic carbonyl compounds can be hydroxylated preferentially ortho, para in contrast to their normal meta directing character.

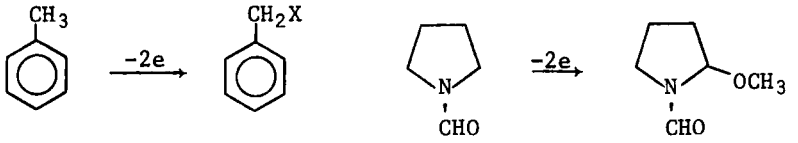
Alkoxylation reactions provide another route to C-O bond formation.[22] This reaction is limited in scope to activated aromatics because of alcohol oxidation, but a number of useful syntheses have come about. Recent papers have, for example, demonstrated that functionalized quinones can be synthesized by blocking the carbonyls in this fashion,[24] and that 4,4-dialkoxy-2-butenoates are available from furfural.[25]



Another useful substitution reaction giving, in this case, Ar-C bonds is aromatic cyanation. Due to the ease of oxidation of cyanide, this reaction is limited to activated aromatics, but direct replacement of hydrogen or RO by cyanide is so uncommon this process should have application.[26]

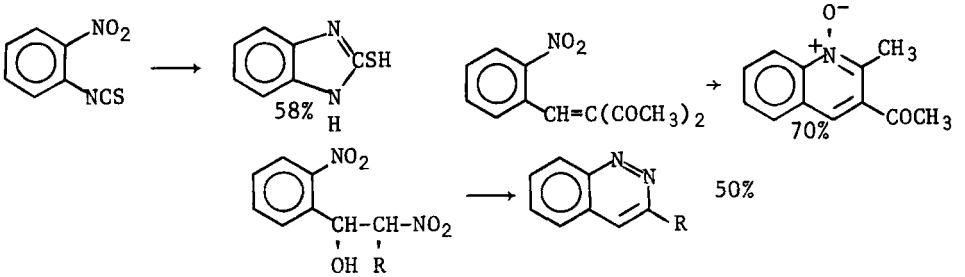


Conversion of aliphatic C-H to C-N or C-O bonds can also be accomplished anodically. Aryl groups, nitrogen and oxygen atoms are relatively easy to oxidize and direct the substitution position α.[24]

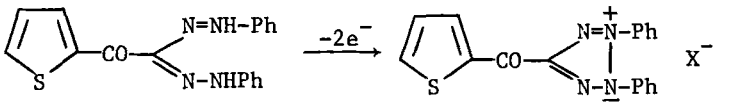


Indeed, the former example illustrates a severe limitation of anodic aromatic substitution. Unactivated aliphatic positions can also be substituted if solvents which are inert to oxidation are employed.[27]

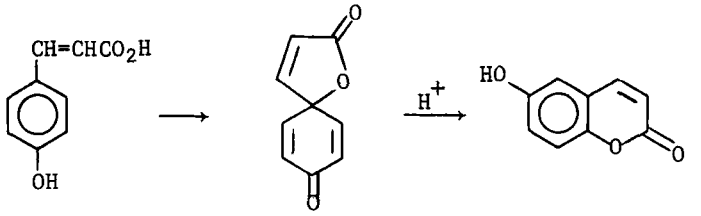
Construction of Heterocycles and Organometallics - Although there are many odd examples of heterocycle formation, especially from anodic processes, there are only a few which were preconceived and which would seem to have generality. Notable among these is a series of cyclization reactions of o-substituted nitroaromatics studied by Lund and coworkers,[28] e.g.,



The tetrazole-formazan conversion can be accomplished oxidatively in very high yield for a number of cases.[29]



In other oxidative processes one simply takes advantage of internal trapping of cationic species by nucleophiles.[30]



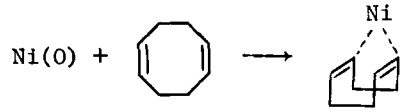
Although studies directed toward the synthesis of organometallics have a limited scope, two interesting and useful types of processes have been developed. A number of routes to alkyl metals have been developed[31] and, indeed, tetraalkyl lead compounds are produced commercially by an electrochemical route. Two reactions are shown here in which organometallics are oxidized. The oxidation releases an alkyl group at a "dissolving" anode producing the desired product.

$$Pb(anode) + CH_3MgCl \rightarrow (CH_3)_4Pb$$

$$Hg(anode) + Al(C_2H_5)_4^- \rightarrow Hg(C_2H_5)_2 + Al(C_2H_5)_3$$

The second general scheme involves interception of reduced metal species formed cathodically. For example:

$$Ni(acac)_2 + 2e^- \rightarrow Ni(0) + 2(acac)^-, \quad acac^- = CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH=\overset{O^-}{\overset{|}{C}}\text{-}CH_3$$



In this particular example reduction of the Ni(II) complex produces Ni(0) which can be trapped by certain ligands. The competing reaction is formation of metallic nickel which is unreactive toward cyclooctadiene. Using this method a number of cyclooctatretaene (COT) have also been formed. Examples are $Ni(COT)$, $Fe(COT)_2$, $Ti(COT)_2$ and $C_2H_5Ti(COT)$.

Summary - The reactions illustrated here have usually been discovered and developed by chemists primarily interested in electrochemistry, not synthesis. Thus, few attempts have been made to electrosynthesize complex molecules or to develop the reactions in the sense that other organic reac-reactions are usually refined for synthetic purposes. The next few years should see this situation change and electrodes will be more widely used as powerful, specific and controllable heterogeneous catalysts.

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Chapter 32. The Use of Stable Isotopes in Medicinal Chemistry

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I. Introduction - Stable isotopes have been used increasingly in recent years in a variety of chemical and biomedical applications. Intensified interest in the use of stable isotopes is reflected by the convening of two international symposia on the subject within the past two years.[1,2] Expanded use of stable isotopes, especially ^{2}H, ^{13}C, ^{15}N and ^{18}O, derives from the development of sophisticated instrumentation (nmr, ir, esr and ms) the greater availability of enriched isotopes,[3] and an espousal on ethical grounds for non-radioactive techniques in human studies, particularly for neonates and pregnant women. One report has appeared on the lack of toxic effects observed in vivo with carbon-13 replacement of carbon-12.[4] The major disadvantages of the use of stable isotopes is the lack of simple, inexpensive instrumentation for detecting the isotopes and the cost of synthesizing the labeled compounds. These and other advantages and disadvantages of using stable isotopes are discussed more thoroughly in ref. 1 and in an earlier commentary.[5]

Two articles appear in 1975 which cover most of the literature on the use of stable isotopes up to that year. The first was a review of studies with deuterated drugs[6] and it also covered the toxicological and therapeutic aspects of deuterium oxide (heavy water). The second article reviewed mass spectrometry.[7] Since the mass spectrometer has been the instrumental tool for most of the research that employs stable isotopes, the article covered many references on stable isotopes as well.

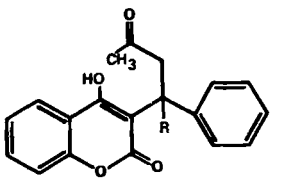
This review is intended to survey those uses of stable isotopes which are of particular importance in medicinal chemistry. These include their use in 1) structure elucidation, 2) studies of drug metabolism, 3) pharmacokinetic analysis and 4) drugs used in therapy. The use of stable isotopes for investigating intermediary metabolism, and their rapidly expanding use in clinical chemistry will not be reviewed since these topics have been covered elsewhere.[1,8] Synthesis and biosynthesis with stable isotopes have also been previously surveyed[9,10] and many articles appear in each issue of the Journal of Labelled Compounds and a few have appeared in Biomedical Mass Spectrometry in the past year. In addition, one recent article has appeared on the use of plants to continously produce stable isotope labeled compounds of pharmacological interest.[11]

II. Use of Stable Isotopes for Structure Elucidation - Mass spectrometry is commonly used in medicinal chemistry to determine the structure of therapeutic agents and their metabolites. The unequivocal interpretation of the mass spectrum for most classes of compounds requires the use of derivatives which are labeled specifically with stable isotopes. For example, the loss of 16 mass units from hydroxamic acids was shown to be due to the loss of the hydroxylamino oxygen by specific labeling with

oxygen-18.[12] The principal modes of fragmentation of N-acyl derivatives of daunosamine, the glycoside moiety of the antitumor antibiotics daunomycin and adriamycin, were determined using specifically deuterated derivatives.[13] A study of the fragmentation pattern of deuterium and oxygen-18 labeled derivatives of cytosine nucleosides established that a major fragmentation process occurred that was absent in the spectra of other nucleosides.[14] The various fragmentation pathways for prostaglandins A, B, E, and F were determined by the use of specific derivitization with d_9-tetramethylsilane.[15]

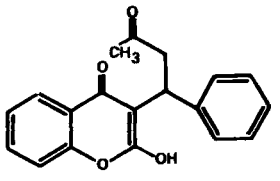
Peptides are becoming increasingly important in medicinal chemistry, and the elucidation of peptide and protein structure has rapidly expanded in the past year. Lithium aluminum deuteride reduction has been used to determine the primary structure of a carboxypeptidase inhibitor from potatoes,[16] and combinations of deuterated and permethylated derivatives have revealed structures of several oligopeptides.[17] The amino acid sequence of other peptides was determined by Edman degradation using p-bromophenylisothiocyanate.[18-20] This technique utilized the ion-doublet arising from ^{79}Br and ^{81}Br to unequivocally identify the p-bromophenylthiohydantoins of the amino acids.

The use of nmr and ir in conjunction with labeling using stable isotopes is important in solving many structural problems which cannot be readily solved by other methods. This is particularly true in the study of dynamic systems where the structure or conformation of a medicinal agent is subject to change. A relevant example in the literature is the work that has been done on the anticoagulant, warfarin (1).[21,22] This compound can potentially exist in solution in several different tautomeric forms. After specifically labeling warfarin in 4-hydroxycoumarin (1), 2-hydroxychromone (2), and the cyclic hemiketal (3) with ^{13}C in the lactone carbonyl group, the tautomeric equilibrium of warfarin was investigated by ^{13}C-nmr analysis. The cyclic hemiketal diastereomers (3) were found to be the predominant tautomers in solution.



1 R = H

4 R = OH



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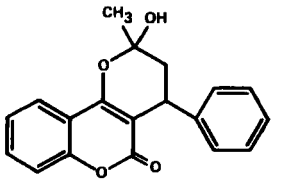

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In other ^{13}C-nmr studies, the effect of pH on the structure of phenobarbital and diphenylhydantoin[23] and on the conformation of amino acids and peptides[24] has been examined using the ^{13}C-enriched drugs.

Dynamic interactions of medicinal agents with biological macromolecules, such as plasma proteins, receptors, or metabolizing enzymes is another area which can be studied by nmr and ir using drugs specifically labeled with stable isotopes. Several examples exist in the biochemical literature and the procedures can be adapted to the study of organic medicinals. For example, amino acid side-chains have been labeled with carbon-13 enriched electrophiles and ^{13}C-nmr was then used to elucidate the reacting side-chain functionality.[25] An extension of this technique would be to use active-site directed enzyme inhibitors, some of which are drugs, to probe the active sites of enzymes. Recently, ^{15}N- and ^{2}H-nmr techniques have been applied to studies of macromolecular structure including work on heme-peptide interactions in hemoglobin,[26] phospholipid-membrane interactions,[27] and the interaction of oxytocin with its protein carrier, neurophysin.[28]

III. Use of Stable Isotopes in Studies of Drug Metabolism and Toxicology - Since the advent of mass spectrometry (ms) coupled with gas chromatography (gc-ms), stable isotopes have been used extensively in the study of drug metabolism. The use of ms in the field of drug metabolism has been reviewed,[29,30] and both reviews cover the use of stable isotopes. There are several ways in which stable isotopes have been used to study drug metabolism and these will be covered individually.

A. Quantification of Drugs and Metabolites - Stable isotope dilution, a technique in which the unlabeled drug is dosed and the labeled substance serves as carrier and internal standard for measurement of the drug and its metabolites in biological fluids, is the most widely used application of stable isotopes. This technique has been comprehensively surveyed[31-33] and need not be detailed here. An international symposium on this subject was held in Ghent, Belgium in June 1976 and the proceedings soon will be published (A. DeLeenheer, ed.). Additional new references appeared throughout 1976 in every issue of Biomedical Mass Spectrometry and most other journals relating to drug metabolism. Three articles of special interest to users of stable isotopes and ms for quantitative purposes are 1) "Limits of Detection of Carbon-13 Labeled Drugs and Their Metabolites in Human Urine",[34] 2) "A Comparison of Unlabeled and Labeled Internal Standards for Quantification by Single and Multiple Ion Monitoring",[35] and 3) "A Review of the Statistical Considerations Involved in the Treatment of Isotope Calibration Data".[36]

Two general methods utilizing the stable isotope dilution technique have been successfully used for the quantification of drugs and their metabolites by ms. The simplest, but least sensitive and specific method, is a non-chromatographic technique that utilizes chemical ionization mass spectrometry (cims) and internal standards labeled with stable isotopes. This method has been used to quantify the antiarrhythmic drugs, lidocaine and quinidine and some of their metabolites, in human plasma samples.[37]

The second, most widely applied method, utilizes the high resolving power of the gas chromatograph and a technique for selected monitoring(mass

fragmentography) of the gas chromatographic effluent. This method allows for the quantification of picogram quantities of both endogenous and exogenous substrates, such as prostaglandins, which otherwise cannot be specifically measured in biological samples. Exhaustive reviews,[38,39] on the uses of this technique have recently been published, and most examples employ stable isotope dilution assays.

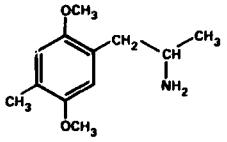
B. Twin-Ion Technique - This technique was used originally to follow the metabolism of chloropromazine, because the ions from the drug and its metabolites which contained chlorine could be easily recognized in a mass spectrum by the natural isotopic pattern of ^{35}Cl and ^{37}Cl.[40] Other investigators soon applied this technique to biotransformation studies of non-halogen-containing compounds by incorporating stable isotopes such as ^{2}H, ^{13}C, ^{15}N and ^{18}O into the compound under investigation. Examples of drugs[41] that have been investigated by this technique include testosterone, nortriptyline,[42] d-propoxyphene,[43] 4-morpholine-2-piperazinothieno [3,2-d] pyrimidine[44] and estrogens.[45] More recently the technique has been used in conjunction with cims to rapidly elucidate the structure of a previously unidentified metabolite of warfarin using a 1:1 mixture of ^{13}C-benzylic warfarin and unlabeled drug.[46] The ion-doublets arising in the chemical ionization mass spectrum of the metabolite helped identify the compound as the benzylic alcohol (4). Similarly, the administration of an equimolar mixture of unlabeled and ^{15}N-labeled phenoxybenzamine to rats, dogs, and man facilitated the identification of urinary metabolites of this antihistamine using gc-cims by virtue of the conspicuous equal-intensity ions in the recorded mass spectra.[47]

Application of cims and the twin-ion technique, using ^{2}H-labeled compounds, has been made in the past year to better define mechanisms of drug toxicity for acetylhydrazine, a hepatotoxic metabolite of the antituberculosis drug isoniazid, and isopropylhydrazine, a hepatotoxic metabolite of the antidepressant drug, iproniazid.[48,49] Highly reactive acylating and alkylating species were generated by the oxidative metabolism of acetylhydrazine and isopropylhydrazine, respectively. These reactive metabolites bound to tissue protein and caused tissue necrosis. The reactive intermediates were trapped using cysteine as an alternate nucleophile and a comparison of the ratio of hydrogen and deuterium in the cysteine adducts to the ratio in the initial substrates showed that the entire acetyl group of acetylhydrazine and the entire isopropyl group of isopropylhydrazine were transferred in the acylation and alkylation process. This suggested the formation of possible cationic or free radical intermediates.

Approximately equimolar mixtures of trideuterated diethylstilbestrol and unlabeled diethylstilbestrol also have been used to study the metabolic activation of diethylstilbestrol to potentially toxic metabolites by rat liver homogenates.[50] Reactive polar metabolites were converted to non-polar metabolites by enzymatic methylation. The ion-doublets arising in the mass spectra of these derivatives guaranteed that these compounds were metabolites of the substrate and also helped to establish their structures.

C. Comparative Metabolism of Enantiomers - Many drugs possess one or more chiral centers and the pharmacological activity of the various isomers may

differ considerably. This difference is often due to stereoselective metabolism of a particular isomer. An elegant method for investigating the comparative metabolism of enantiomers is to administer a 'pseudoracemate' in which one enantiomer is labeled with a stable isotope. The enantiomeric ratios for the drug and its metabolites can then be determined while the activity of the drug is being monitored. This technique has been applied to investigations of the differential metabolism of the enantiomers and racemate of the psychotomimetic amine, 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (5),[51-53] and amphetamine (6).[54] Results in both cases showed that the racemate and individual enantiomers are metabolized differently. For example, comparison of separate incubations of R-amphetamine (R-6) with those of S-amphetamine-d_3 (S-7) and of S-amphetamine (S-6) with those of R-amphetamine-d_3 (R-7) showed that larger amounts of the N-hydroxyamphetamines, 8 and 9, and the alcohols, 10 and 11, were formed from the R-enantiomers. However, when pseudoracemic mixtures of R-6/S-7 or R-7/S-6 were incubated the metabolites were preferentially formed from the S-isomer. It was concluded that S-amphetamine or one of its metabolites inhibited the metabolism of the R-enantiomer.



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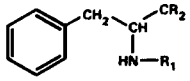
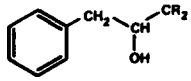


	R_1	R_2
6	H	H
7	H	^2H
8	OH	H
9	OH	^2H



	R_2
10	H
11	^2H

Simultaneous measurements of plasma levels of deuterated and unlabeled d- and l-propoxyphene revealed that the plasma levels of the more analgesically active d-isomer were higher and the half-life was longer.[55] On the other hand, simultaneous measurements of (+)- and (-)-propranolol indicated no differences in the rate of elimination of the isomers in dogs.[56] Finally, the metabolism of carbon-13 labeled R- and S-α-methyldopa has revealed a high degree of stereoselectivity favoring the S-enantiomer for both transport across the blood-brain barrier and brain decarboxylase activity.[57]

D. Use of Isotopes in Studying Enzymatic Mechanisms of Drug Metabolism - The most widely used stable isotope in determining enzymatic mechanisms is deuterium because of the large mass difference between it and hydrogen.[58] This mass difference leads to differences in the zero-point energy of a bond to deuterium and hydrogen which in turn leads to isotope effects on the rates of metabolic processes where cleavage of a C-H vs. C-D bond is rate-determining. Because the oxidative metabolism of drugs usually involves such a process, there are many examples of isotope effects on the metabolism of drugs.[6] Two recent articles have appeared on theoretical treatments of kinetic isotope effects which should be of help in designing more interpretable deuterium isotope experiments.[59,60]

Aromatic hydroxylations do not in general exhibit kinetic deuterium isotope effects since the rate-determing step is the heterolytic cleavage of a carbon-oxygen bond of an intermediate arene oxide.[61] Small isotope effects have been found and may have been due to secondary deuterium isotope effects. Such effects have been observed for peracid-type oxidations.[62] However, significant isotope effects (k_H/k_D = 1.3-1.75) are associated with the aromatic meta-hydroxylation of benzene substituted with electron-withdrawing substituents,[63] possibly indicating a different oxygenation mechanism or change in rate-determing step.

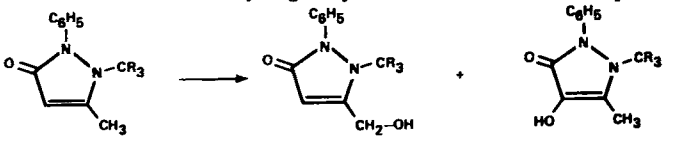
Other common pathways of drug metabolism include oxidative O- and N-dealkylation. Deuterium isotope effects of ~2 have been observed for the O-demethylation of a variety of anisoles.[64-66] Small isotope effects (k_H/k_D ~ 1.3) have been observed for the N-demethylation of amines.[67,68] The effects are exclusive of secondary effects on amine basicity as determined in one of these studies.[68] Studies of the oxidative dealkylation by liver microsomes of deuterated N-alkylamphetamines has indicated that α-carbon oxidation was responsible for dealkylation of the S-enantiomers, whereas N-oxidation was probably the rate-determining step for dealkylation of the R-enantiomers.[69] Several other studies have been carried out on C- vs. N-oxidation of amphetamines using atmospheres of $^{18}O_2$ and deuterium labeling, and mechanisms have recently been reviewed.[70]

Two examples of unusually large deuterium isotope effects in biological systems have been observed. The oxidative O-demethylation of trideuteromethoxy anisole showed an isotope effect of ~10 *in vitro*,[65] and the oxidation *in vivo* of cotinine, a nicotine metabolite, to 3-hydroxycotinine showed an isotope effect of ~7 when 3,3-dideuterio-cotinine was used.[71]

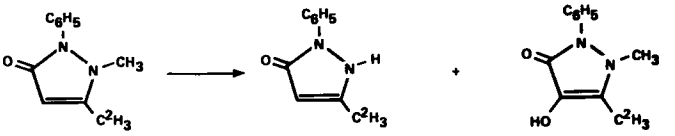
A recent study has employed deuterium labeling to show that the mechanism for the oxidative N-demethylation of nicotine may involve two modes of breakdown for a proposed carbinolamine intermediate, dealkylation with formaldehyde formation and dehydration to an iminium ion.[72] The formation of such an sp^2-hybrid intermediate may help to explain why both a primary and substantial β-secondary deuterium isotope effect were observed for the N-deethylation of the antiarrhythmic agent, lidocaine.[73] In contrast, only a primary isotope effect was observed on the rate of oxidative O-deethylation of deuterated analogs of the analgesic, phenacetin.[74] These results indicate differences in the mechanism of oxidative O- and N-dealkylation. A final example of the use of secondary deuterium isotope effects in studying enzymes involved in drug metabolism revealed an SN-2-like transition state for the transfer of a methyl group catalyzed by catechol-O-methyl transferase.[75]

E. Switching of Metabolic Pathways with Deuterium - The magnitude of deuterium isotope effects observed in drug metabolism varies considerably and apparently is influenced by the availability of alternate pathways of metabolism. Recent evidence suggests that if a drug is metabolized by multiple alternate pathways, the metabolism may be shifted by deuterium labeling at a site of metabolism.[76] Studies both *in vivo* and *in vitro* on the effect of deuterium substitution on N-demethylation and on the conversion of a methyl group to a hydroxymethyl group have been investigated

for antipyrine, and caffeine.[76] In both cases examined, deuterium substitution in one position switches metabolism to an alternate position. For example, the metabolism of antipyrine (12), N-C^2H_3-antipyrine (13), and 3-C^2H_3-antipyrine (14) were compared. The major urinary metabolites of 12 and 13 were 4-hydroxy- and 3-hydroxymethylantipyrine. In contrast, the major metabolites of 14 were 4-hydroxyantipyrine and N-demethylantipyrine; the metabolism of antipyrine was switched by deuterium substitution from hydroxylation of the methyl group on C-3 to N-demethylation.



12 R = H
13 R = ^{2}H



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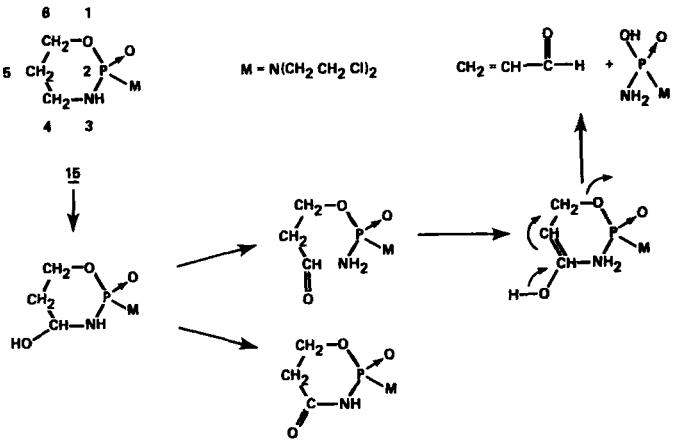
These results indicate that the metabolic disposition and thereby the pharmacological activity, whether efficacious or toxicological, could be modified by selective substitution of deuterium for hydrogen. A new combination antibacterial contains 3-fluoro-D-alanine-2-d which is an excellent example of selective deuteration to enhance the pharmacological activity of a therapeutic agent.[77] The metabolism *in vivo* of 3-fluoro-D-alanine is reduced several-fold by substitution of deuterium for hydrogen on the 2-position without loss of antibacterial activity. This enhances the therapeutic index of the compound because metabolism of 3-fluoro-D-alanine leads to the formation of an inactive antibacterial and fluoride which is nephrotoxic.

F. Use of Isotopes to Study Reactive Metabolites - Most drugs are metabolized to inactive and sometimes more active metabolites. However, a few drugs are also metabolized to reactive, often toxic, metabolites which bind to tissue macromolecules (RNA, DNA, and proteins) and can lead to carcinogenesis, mutagenesis, or tissue necrosis.[78] Stable isotope labeling has been used to examine mechanisms in the formation of these reactive metabolites. Two examples have already been presented in Section IIIB using the twin-ion technique (ref. 48-50). Mechanisms for the covalent binding of arylating metabolites of the widely used analgesics p-hydroxyacetanilide (acetaminophen) and p-ethoxyacetanilide (phenacetin) to hamster microsomal protein have also been investigated using $^{18}O_2$.[79] The mechanism of reactive metabolite formation from acetaminophen was found to be different than that for phenacetin *in vitro*.

Anti-cancer drugs such as cyclophosphamide (15), aniline mustard, and nitrosoureas are transformed to reactive metabolites which are the toxic species required for their anti-cancer activity.[80] Experiments with selectively deuterated analogs of these drugs has distinguished which pathway, among several alternative pathways of metabolism, is responsible for anti-tumor activity. For example, a deuterium isotope effect was observed for the formation of 4-ketocyclophosphamide (16), formed by the oxidation of the carbon alpha to the phosphoramide nitrogen, but there was no isotope effect on the anti-tumor activity. However, there was a marked effect on the subsequent β-elimination reaction and consequent decrease in anti-tumor activity by deuterium substitution at C-5. Thus, the formation of acrolein and phosphoramide mustard is rate determining for the anti-tumor activity of cyclophosphamide.



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The carcinogenicity of dimethylnitrosamine and 4-nitrosomorpholine was reduced by deuterium substitution for hydrogen on carbon atoms alpha to the amino nitrogen.[81,82] Consistent with the hypothesis that alpha-carbon oxidation is required for reactive metabolite formation from nitrosamines, there is a substantial primary deuterium isotope effect (k_H/k_D = 3.8) on the rate of dimethylnitrosamine N-demethylation.[83] Specific deuteration of 3-methylcholanthrene, a potent polycyclic hydrocarbon carcinogen, showed that oxidation of the 1-carbon atom is critical in the tumor-initiating process in mouse skin.[84]

Stable isotopes other than deuterium have also been used in some novel approaches for studying reactive metabolite formation. Benzo[a]pyrene, another potent polycyclic hydrocarbon carcinogen, was incubated with co-factors and rat liver microsomes in an atmosphere of $^{17}_{8}O_2$ to investigate whether or not 6-oxybenzo[a]pyrene radical was formed. Electron spin

resonance analysis indicated that very small or undetectable amounts were formed enzymatically. On the contrary, a radical was formed non-enzymatically by air oxidation of benzo[a]pyrene after extraction of the metabolites from the incubation mixture. Infrared analysis was used to determine that the increase in carboxyhemoglobin formation subsequent to the administration of methylene chloride-^{13}C to rats, is caused by its metabolism to carbon monoxide.[86]

IV. Use of Stable Isotopes in Pharmacokinetics - The quantification of drugs and their metabolites by isotope dilution using gc-ms has been applied by several investigators to study the pharmacokinetics of drug metabolism, distribution, and excretion. The basic approach used in these investigations is illustrated by studies with amphetamine and phentermine;[87] caffeine, mephobarbital, secobarbital and antipryine.[88] More recently, two elegant adaptations of the general procedure have been used to determine absolute bioavailability[89,90] and steady state kinetics.[91,92]

The absolute bioavailability of N-acetylprocainamide was determined by administering intravenously the ^{13}C-labeled drug at the same time the unlabeled drug was given orally.[89,90] Plasma levels and urinary excretion of both compounds were quantified with the use of a d_5-labeled internal standard. Oral absorption was found to be within a range of 75% to 90% in three human subjects and peak plasma levels were attained in 45-90 minutes. The slow absorption rate of N-acetyl-procainamide, coupled with a long half life for elimination plus therapeutically effective plasma levels, make the compound attractive as an oral anti-arrhythmic agent. The stable isotope procedure that was used to determine these parameters offered the advantage of determining the bioavailability and first-pass effects of the drug from a single study by the analysis of only one set of blood samples, and permitted the determination of the kinetics of drug distribution and elimination at the same time that the absorption was under investigation.

The steady state kinetics of methadone[91] and propoxyphene[92] were investigated by substituting the daily dose of the unlabeled compound by a deuterium labeled derivative, and following the plasma half-lives of both drugs. The half-lives of the deuterated derivatives were found to be three to seven times shorter than the unlabeled drugs, which had been administered chronically. These results suggested that deep pools of tissue-bound, unlabeled drug existed which were not readily available to the deuterated derivatives. This experiment illustrates the importance of this technique for investigating the complexities of steady state pharmacokinetics.

In summary, stable isotopes are being effectively used in many areas which either directly or indirectly affect research in medicinal chemistry. Hopefully, this review has highlighted those areas of interest to most medicinal chemists.

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