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New Directions in the Chemistry of Natural Products: The Organic Chemist as a Pathfinder for Biochemistry and Medicine*

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A. *Introduction*

'*A large part of chemistry deals with change.* The essential work of the chemist is not just to observe change as it occurs in the world, but to control and change the material world according to man's needs. The chemist not only isolates naturally occurring drugs; he also determines their structures, synthesizes them, seeks to understand how they function, and improves on them. He not only observes catalysis in living systems; he creates new catalysts. To a great extent, chemistry is dedicated to control of the material world; it is a highly practical discipline. In the practical world, basic research provides the discoveries on which advances in science and industry must draw to meet future challenges. Fortunately, the science of chemistry is in a period of rapid growth, in which numerous opportunities for scientific and technical advance offer promise of a future as spectacularly successful as its past'[1]. We are now inclined to view this optimistic assessment made 6 years ago with some scepticism. We have become somewhat uncertain about the significance of simplistic viewpoints of the past. As MAX WEBER prophesied: 'Science also prepares to change its standpoints and its conceptual apparatus in order to look down from the heights of thought on the current of events'. We are looking for new constellations of values that determine the choice of problems which are regarded as significant. What has benefited the chemist most in the last 20 years is the vastly expanded and more sophisticated armamentarium that he can command in his attempt to solve problems which could not be attacked by classical methods.

Toad venoms, butterfly pigments, calabash curare were the topics of his distinguished laboratory when in the late thirties my mentor, HEINRICH WIELAND (1877–1957), asked me to take up the poison of the death cap (*Amanita phalloides*) for my thesis. Over 30 years later, from the vantage point of hindsight, we recognize the importance of amanitin[2] for the selective inhibition of specific RNA polymerases[3] or the connection between pterins, i.e. butterfly pigments, folic acid and aryl hydroxylases[4]. What has changed in all these years for the natural products chemist, especially one working in a mildly mission-oriented environment, is the realization that isolation, structure and even synthesis of new natural principles are just prologue. The drama does not unfold until these children of nature are taken by man and are returned to participate in the dynamics of life in other systems.

B. *Histrionicotoxin, the first acetylenic venom*

Acting on the principle 'reculer pour mieux sauter' I should like to present a story so new that only isolation and structure can be discussed. The venom itself has not yet come to life, say, as a CNS agent or as a pharmacological tool. However, its structure is fascinating and has no precedent[5].

At a time when body painting has become part of the psychedelic scene, the Colombian frog *Dendrobates histrionicus* offers, in its many species varieties, the most challenging examples of decorative skin colors and patterns (Color Plate). Even black and white reproductions (Figures 1 and 2) give some intimation of nature's inventiveness. Earlier we had looked at *Dendrobates pumilio* (bottom right on Color Plate) and had isolated pumiliotoxin C, IV[6], a bicyclic analog of R(+)-coniine (II), whose absolute stereochemistry at C-2 the former may or may not share[7]. The question

* 13. Paul Karrer Lecture, presented 30 June 1971, in the Aula of the University of Zurich. German title: Neue Ziele in der Naturstoffchemie: Der organische Chemiker als Wegbereiter der Biochemie und Medizin.

[1] *Chemistry: Opportunity and Needs*, a report on basic Research in US Chemistry (known as 'The Westheimer Report') (National Academy of Sciences, Washington, D.C. 1965), p. 1.

[2] TH. WIELAND, Science *159*, 946 (1968); cf. TH. WIELAND, *Der organische Chemiker und die Molekularbiologie* (Jahrbuch der Max-Planck-Gesellschaft zur Förderung der Wissenschaft, 1970), p. 146.

[3] L. FIUME and TH. WIELAND, FEBS Letters *8*, 1 (1970). – Cf. P. A. HORGEN and D. H. GRIFFIN, Proc. natn. Acad. Sci., USA *68*, 338 (1971).

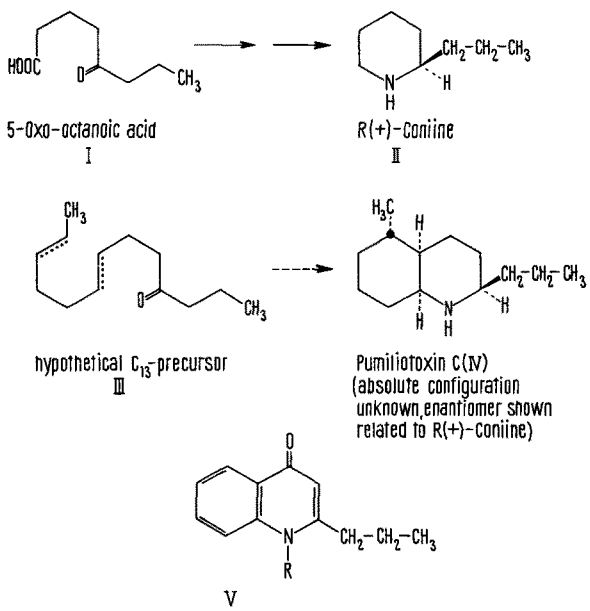
[4] W. SHIVE, in *Comprehensive Biochemistry* (Eds. M. FLORKIN and E. H. STOTZ; Elsevier, Amsterdam, London, New York 1963), vol. 11, p. 82. – L. JAENICKE and C. JUTZBACH, in *Progress in the Chemistry of Organic Natural Products* (Ed. L. ZECHMEISTER; Springer Verlag, Wien 1963), vol. 21, p. 183. – S. KAUFMAN, Pharmac. Rev. *18*, 61 (1966). Biochemistry, *10*, 2330 (1971).

[5] J. W. DALY, I. L. KARLE, C. W. MYERS, J. W. WATERS, T. TOKUYAMA and B. WITKOP, Proc. natn. Acad. Sci., USA, *68*, 1870 (1971).

[6] J. W. DALY, T. TOKUYAMA, G. HABERMEHL, I. L. KARLE and B. WITKOP, Ann. Chem. *729*, 198 (1969).

[7] W. A. AYER and T. E. HABGOOD, in *The Alkaloids* (Ed. R. H. F. MANSKE; Academic Press, New York, London 1968), vol. 11, p. 473.

Fig. 1. *Dendrobates histrionicus*, the color variety on the South bank of the Rio Docordó, a tributary of the lower Rio San Juan.



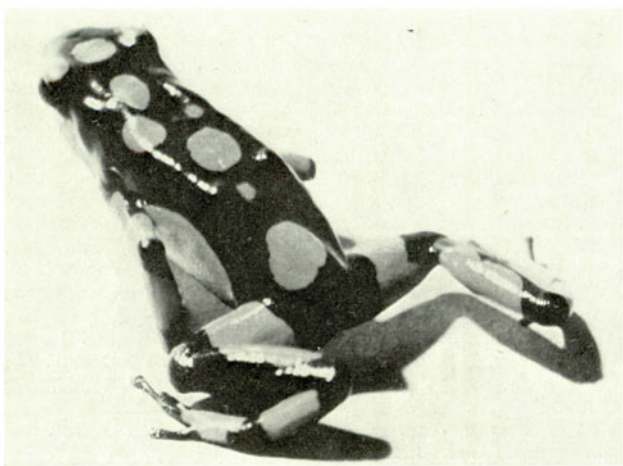
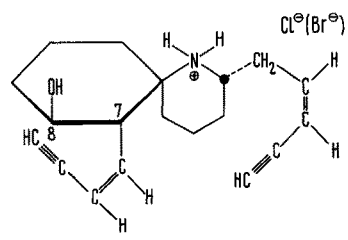


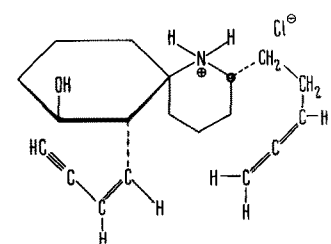
Fig. 2. *Dendrobates histrionicus*, the color variety from the North bank of the Rio Docordó, a tributary of the lower Rio San Juan.

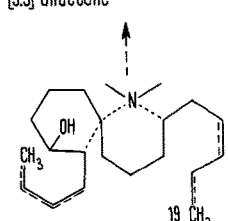
of stereochemistry as well as of the somewhat intricate synthesis of this novel *cis*-decahydroquinoline (IV) are now under investigation in collaboration with the laboratory of G. Habermehl in Darmstadt. The biosynthesis may utilize a C_{13}-straight-chain precursor (e.g. III) in the same way as 5-oxo-octanoic acid (I) is utilized in hemlock[8]. Quinolones with *n*-propyl substituents in the 2-position, such as V, are known to occur in plants[9].

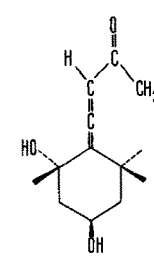
Illustrative of the modern approach to a natural product investigation, the study of histrionicotoxin required a fine integration of organization and timing (Table I).

Not only was histrionicotoxin established as (2*p*R, 6S, 7*p*S, 8*a*S)-7-(*cis*-1-buten-3-ynyl)-8-hydroxy-2(*cis*-2-penten-4-ynyl)-1-azas*piro*[5.5]undecane, but by the comparison of the effects of anomalous dispersion on the magnitudes of F_{hkl} and $F_{\bar{h}\bar{k}\bar{l}}$ of pairs of reflections[10] of the hydrobromide the absolute configuration, as shown in VI, was ascertained. Although about 450 acetylenic nonbasic plant products are known[11], this is the first isolation of a (basic) acetylenic derivative from an animal. The presence of 8-*cis*-dihydromatricaria acid in the secretion of the soldier beetle (*Chauliognathus lecontei*)[12] is attributed to uptake from the diet









[8] E. LEETE and J. OLSON, Chem. Commun. *1970*, 1651.
[9] A. M. DUFFIELD and P. R. JEFFERIES, Austral. J. Chem. *16*, 292 (1963).
[10] J. M. BIJVOET, A. F. PEERDEMAN and A. J. VAN BOMMEL, Nature, Lond. *168*, 271 (1951).
[11] F. BOHLMANN and H. J. MANNHARDT, in *Progress in the Chemistry of Organic Natural Products* (Ed. L. ZECHMEISTER; Springer Verlag, Wien 1957), vol. 14, p. 1.
[12] J. MEINWALD, Y. C. MEINWALD, A. M. CHALMERS and T. EISNER, Science *160*, 890 (1968).

Colour plate. Neotropical Frogs of the Genus Dendrobates. From left to right, beginning at the top: 1. Color variety of *Dendrobates histrionicus* from Guayacana, Departemento Nariño, Colombia; 2, 3, 4 and 5. Color varieties of *Dendrobates histrionicus* from the Rio San Juan, Departemento Chocó, Colombia; 6. Color variety of *Dendrobates pumilio*. *D. histrionicus* specimens are 35–40 mm long, while *D. pumilio* is approximately 20 mm in length.

Table I. Organization and time schedule for histrionicotoxins

Investigator	Activity	Time schedule
CHARLES W. MYERS, Herpetologist, American Museum of Natural History, New York	Location and classification of *Dendrobates histrionicus*	6th expedition to the jungles of Western Colombia
JOHN W. DALY, Laboratory of Chemistry (LC) National Institute of Arthritis and Metabolic Diseases (NIAMD)	Collection, skinning and extraction of 800 frogs at the natural habitat	6 weeks
JAMES A. WATERS, LC, NIAMD	Fractionation of extracts, separation of active principles	4 weeks
TAKASHI TOKUYAMA, Osaka City University, Japan	Growing of single crystals (Ref.[5])	8 weeks
ISABELLA KARLE, Lab. for the Structure of Matter, Naval Research Laboratory, Washington	Relative configuration by symbolic addition procedure (Ref.[5])	1 week
	Absolute configuration by anomalous dispersion (Ref.[5])	1 week
E. J. COREY, Harvard University	(Computer-assisted[a]) synthesis (13 steps)	In progress
E. X. ALBUQUERQUE, State University of New York at Buffalo	Preliminary pharmacology	In progress

[a] Cf. E. J. COREY and W. TODD WIPKE, Science *166*, 178 (1969).

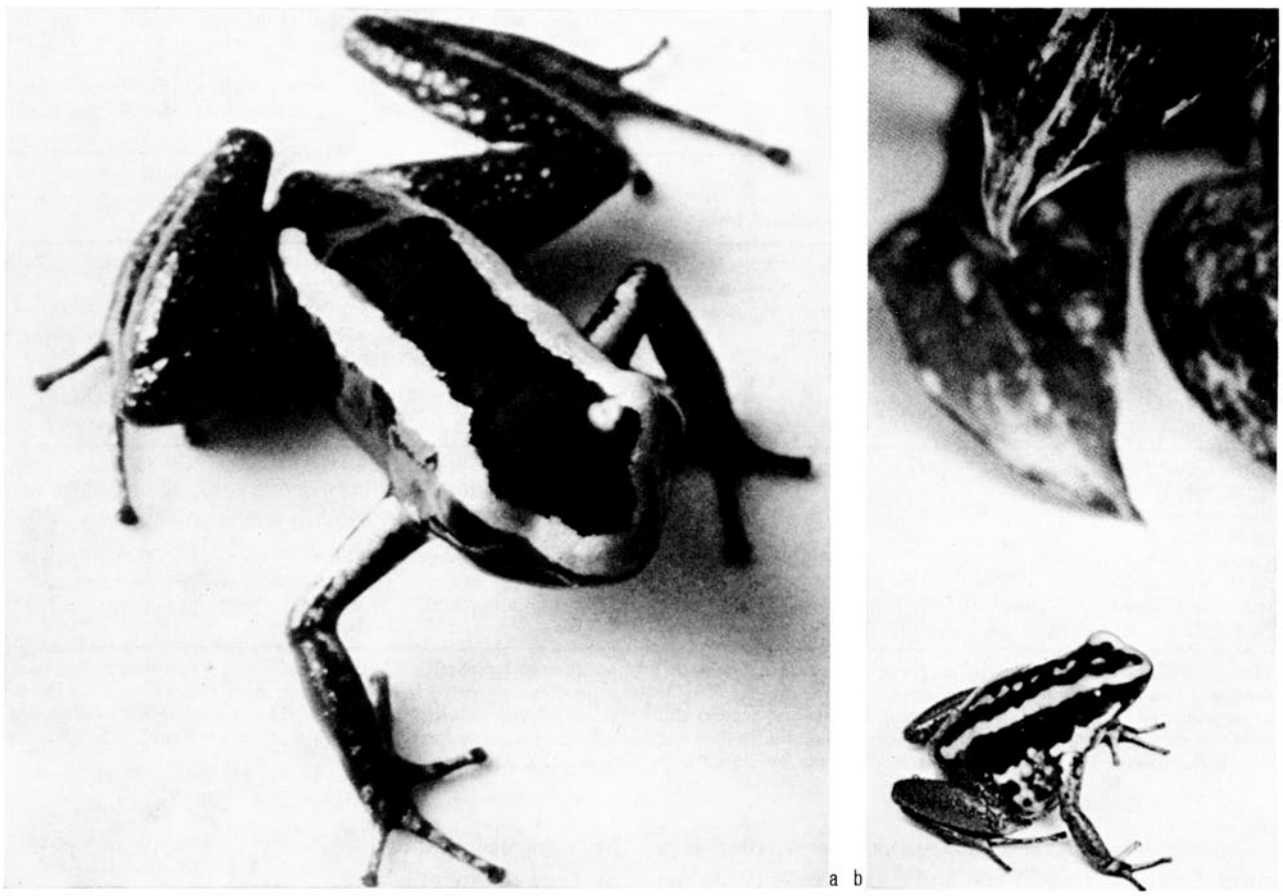


Fig. 3a. The Colombian poison arrow frog, *Phyllobates aurotaenia*. The two dorsal stripes are bright sulfur-yellow contrasting strongly with the black body color.
Fig. 3b. The frog *Phyllobates vittatus* from Costa Rica not only resembles *Phyllobates aurotaenia* from Western Colombia but also contains batrachotoxin.
Photography: GARY LAURISH, Washington, D. C.; Courtesy of Science.

which consists of composites containing acetylenes. That histrionicotoxin might come from the frog's diet of insects that live on similar plants is a rather unlikely chain of events.

In the congener dihydro*iso*histrionicotoxin (VII) the 2-(*cis*-1-buten-3-ynyl) side chain is modified: the *cis*-olefin is reduced and the terminal acetylene is now isomerized to an allene, again the first observation of a basic allene in an animal species. The secretion of the flightless grasshopper, *Romalea microptera*, contains the (neutral) allenic sesquiterpene IX[13]. In a

[13] J. MEINWALD and L. HENDRY, Tetrahedron Letters *1969*, 1657.

Table II. Organizational and chronological survey of the batrachotoxin problem

Investigators	Activity	Date	References
FRITZ MÄRKI, LC, NIAMD Marté Latham	Collection of 8000 *Phyllobates aurotaenia*, 1960–1970	5 expeditions, 1960–1970, each 1–2 months, skinning, extraction, fractionation	F. MÄRKI and B. WITKOP, Experientia *19*, 329 (1963).
JOHN DALY, LC, NIAMD	First homogeneous extracts, microchemical characterization	Collection of a total of 8000 *Phyllobates aurotaenia*	M. LATHAM, National Geographic *129*, 682 (1966).
KLAUS BIEMANN, MIT, Cambridge, Mass.	First mass spectra ('M⊕' 399)	1964	J. W. DALY, B. WITKOP, K. BOMMER and K. BIEMANN, J. Am. chem. Soc. *87*, 124 (1965).
TAKASHI TOKUYAMA, Osaka City Univ., Japan	First crystals (*p*-bromobenzoate), partial synthesis, 'super-batrachotoxin'	1967–1968	T. TOKUYAMA, J. W. DALY and B. WITKOP, J. Am. chem. Soc. *91*, 3931 (1969).
ISABELLA KARLE and JEROME KARLE, Naval Research Laboratory, Washington, D.C.	Symbolic addition procedure	1968	I. L. KARLE and J. KARLE, Acta Cryst. *B25*, 428 (1969);
	Absolute configuration	1970	R. D. GILARDI, Acta Cryst. *B26*, 440 (1970).
EDSON X. ALBUQUERQUE, Principal Investigator, State University of New York at Buffalo	Pharmacology and neurophysiology in many different systems	1968 – present	J. E. WARNICK, E. X. ALBUQUERQUE and F. M. SANSONE, J. Pharmac. exp. Ther. *176*, 498 (1971).
H. WEHRLI, Laboratory of Oskar Jeger, ETH, Zurich	Total synthesis (3-*O*-methyl-17α,20ξ-tetrahydrobatrachotoxinin A)	1968–1971	W. GRAF, H. BERNER, L. BERNER-FENZ, E. GÖSSINGER, R. IMHOF and H. WEHRLI, Helv. chim. Acta *53*, 2667 (1970) and in press.

Table III. Properties of the cardiotoxic alkaloids isolated from the Colombian poison arrow frog, *Phyllobates aurotaenia*[a]

Alkaloid	Toxicity LD_{50} s.c. in mice (μg/kg)	Approximate amount/frog (μg)	Pure compound isolated from 5000 frogs (mg)	Mass spectrum apparent molecular ion (mass)	UV-spectrum nm	ε	IR-spectrum (cm^{-1})	Ehrlich reaction[b] DMAB	DMAC	Rf's[c]
Batrachotoxin (M.W. 538)	2	20	11	$C_{24}H_{33}NO_4$ (399)	234 267	9200 5100	1690	red	blue	0.52
Homobatrachotoxin (M.W. 552)	3	10	16	$C_{24}H_{33}NO_4$ (399)	234 264	9800 5100	1690	red	blue	0.57
Pseudobatrachotoxin (M.W. unknown)	–	20	1[d]	$C_{24}H_{33}NO_4$ (399)	End absorption		–	none	none	0.54
Batrachotoxinin A (M.W. 417)	1000	30	47	$C_{24}H_{33}NO_5$ (417)	End absorption		no carbonyl	none	none	0.35

[a] Isolation consisted of concentrating methanolic skin extracts in vacuo, followed by partition between chloroform and water. The basic principles were extracted from the chloroform layer into 0.1 *N* HCl. After basification with 1 *N* NH_4OH, they were reextracted into chloroform. Subsequent purification was carried out by preparative thin-layer or column chromatography on silica gel. [b] DMAB = dimethylaminobenzaldehyde; DMAC = dimethylaminocinnamaldehyde. [c] Silica gel thin-layer chromatoplates, chloroform-methanol, 7:1, detection with sulfuric acid. [d] Most of pseudobatrachotoxin is converted to batrachotoxinin A during purification.

schematic way histrionicotoxin can be written as a derivative of a straight chain precursor with 19 carbon atoms (or a modified prostaglandin undergoing decarboxylation). Whether the frog utilizes such an approach or the more traditional pathway leading, e.g. to the related erythrina skeleton, is a moot question at present.

The close proximity of the two heteroatoms, N-1 and the hydroxyl at C-8, make histrionicotoxin a potential candidate for cholinergic activity or possible interaction with cholinergic or other receptor proteins[14]. The use of natural products to probe the nature of receptor sites for neurohumoral transmitters, e.g. of bicuculline for the receptor of GABA (γ-aminobutyric acid)[15], or of the destruction of mammalian motor nerve terminals by black widow spider venom[16] is a timely and significant development.

C. *Batrachotoxin, a new tool for sodium transport studies in electrogenic membranes*

While the solution of the histrionicotoxin problem was measurable in terms of weeks, the work on the

[14] J.-P. CHANGEUX, M. KASAI and C. Y. LEE, Proc. natn. Acad. Sci., USA *67*, 1241 (1970). – R. MILEDI, P. MOLINOFF and L. T. POTTER, Nature, Lond. *229*, 554 (1971).

[15] D. R. CURTIS, A. W. DUGGAN, D. FELIX and G. A. R. JOHNSTON, Nature, Lond. *226*, 1222 (1970).

[16] M. OKAMOTO, H. E. LONGENECKER JR. and W. F. RIKER JR., Science *172*, 733 (1971).

related Colombian poison arrow frog, *Phyllobates aurotaenia*, extended over more than 10 years (Table II).

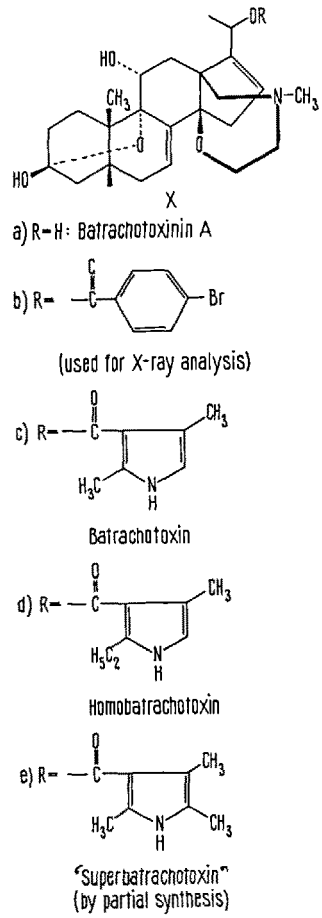
Among the frog venoms[17] the most interesting chemically, as well as pharmacologically, is the steroidal alkaloid *batrachotoxin* (Xc). It is found, together with the equally active homobatrachotoxin (Xd), the unstable pseudobatrachotoxin of unsolved structure, and the much less toxic batrachotoxinin A (Xa), in the skin of the small brightly colored frog (Figure 3a) which the natives call kokoi; it is also found in *Phyllobates vittatus* from Costa Rica (Figure 3b). Table III summarizes the properties of these venoms.

Investigation of batrachotoxin was handicapped by the paucity of material and by its lability. The skin of an adult frog of 3 cm length contains only 80 μg of toxic congeners. The frog, which is found in a rather inaccessible region of Colombia (Figure 4) was difficult to obtain in large numbers; however, in the course of 4 expeditions, approximately 8000 specimens were collected. Methods were developed for the isolation and separation of the active principles, which minimized losses resulting from their great labilities.

Preliminary investigation showed these compounds to be weak bases with a pK_a of approximately 7.5. According to interpretations of high resolution mass spectrometry batrachotoxin and homobatrachotoxin are steroidal alkaloids with the empirical formula $C_{24}H_{33}NO_4$. The much less toxic batrachotoxinin A, on the basis of mass spectral data, contains the additional elements of water in its molecular ion of $C_{24}H_{35}NO_5$. Since the compounds are weakly basic and since the mass spectra indicated only one nitrogen atom, it was quite a surprise when, in the course of microchemical investigation, it was discovered that batrachotoxin and homobatrachotoxin gave a strong positive Ehrlich test, indicative of the presence of a pyrrole moiety. In view of the evidence, the conclusion was inevitable that the basic nitrogen in (homo)batrachotoxin was part of a real or potential pyrrole ring.

A crystal suitable for X-ray analysis was finally obtained in 1967, when Dr. TOKUYAMA succeeded in preparing a *p*-bromobenzoate of batrachotoxinin A, the least toxic of the congeners. X-ray analysis of a tiny crystal of this derivative by the 'symbolic addition procedure' of JEROME and ISABELLA KARLE established its structure as Xb.

With the structure of one of the bases now known, reexamination and reinterpretation of the physical and spectral properties of (homo)batrachotoxin led to the elucidation of structure of the actual venom: when the mass and NMR-spectra of batrachotoxinin A were compared with those of (homo)batrachotoxin, the presence of a common steroid moiety in all of these bases became apparent. Batrachotoxin and homobatrachotoxin, however, both exhibited UV-spectra with λ_{max} at 234 and 264 nm, indicative of a conjugated system, an IR-absorption band at 1690 cm^{-1}, typical of a carbonyl group or perhaps a vinyl ether, and, of course, the positive Ehrlich reaction due to a (potential) pyrrole system. In addition, NMR-spectra showed that batrachotoxin contained 2 methyl groups and homobatrachotoxin, one methyl and one ethyl group not evident in the NMR-spectrum of batrachotoxinin A. It was impossible to rationalize structures for (homo)batrachotoxin solely in terms of a C_{24} steroid structure



closely related to batrachotoxinin A. The inescapable conclusion was that the true molecular ion had so far escaped detection in the mass spectra of (homo)batrachotoxin and that these compounds contained the steroid system of batrachotoxinin A plus another moiety responsible for the ultraviolet chromophore, the carbonyl band, the pyrrole reactions and the additional methyl and ethyl groups. It was postulated that this moiety consisted of a dimethylpyrrolecarboxylate ester in the case of batrachotoxin and an ethylmethylpyrrolecarboxylate in the case of homobatrachotoxin.

[17] J. W. DALY and B. WITKOP, Mem. Instituto Butantan, Simp. Int. *33*, 425 (1968). – J. W. DALY and B. WITKOP, in *Venomous Animals and their Venoms* (Eds. W. BÜCHERL and E. BUCKLEY; Academic Press, Inc., New York 1971), vol. 2, p. 497.

The mass spectra of batrachotoxin and homobatrachotoxin did show additional low-mass nitrogen-containing fragments not present in the spectra of batrachotoxinin A. These fragments, for example, $C_8H_{11}NO_2$ in homobatrachotoxin and $C_7H_9NO_2$ in batrachotoxin, could well have arisen from an ethylmethylpyrrolecarboxylic ester or a dimethylpyrrolecarboxylic ester, respectively. It now remained to prove that batrachotoxin and homobatrachotoxin were, indeed, dialkylpyrrolecarboxylates of batrachotoxinin A.

The mass spectrum of batrachotoxin was reexamined and great attention was given to detecting the true molecular ion. As predicted for a dimethylpyrrolecarboxylate of batrachotoxinin A, a very weak molecular ion was found at *m/e* 538. Batrachotoxin was then hydrolyzed in base. A low yield of a basic alcohol identical with batrachotoxinin A was obtained.

The next task was to establish the position of esterification and the nature of the dialkylpyrrolecarboxylate moiety. Comparison of the NMR- and mass spectra of (homo)batrachotoxin, as well as of batrachotoxinin A and of its 20α-*p*-bromobenzoate, clearly established that the position of esterification in (homo)batrachotoxin was the 20α-hydroxyl group. The resonance peak for the 20β-hydrogen in batrachotoxinin A appeared at 4.58 δ, while in (homo)batrachotoxin and the 20α-*p*-bromobenzoate of batrachotoxinin A, it is shifted downfield by 1.3 ppm, a change compatible with esterification of the 20α-hydroxyl group.

The ring substitution pattern of the dialkylpyrrolecarboxylate moiety was now investigated. A comparison of UV-spectra of ethyl pyrrolecarboxylates with those of (homo)batrachotoxin demonstrated the presence of a pyrrole-3-carboxylate in these alkaloids. The position of the 2 alkyl substituents was determined by NMR-spectroscopy by the use of 2 solvents and by comparison of the shifts in methyl resonance of (homo)-batrachotoxin with that of ethyl dimethylpyrrole-3-carboxylates. *Batrachotoxin was shown to be batrachotoxinin A 20α-2,4-dimethylpyrrole-3-carboxylate and homobatrachotoxin to be batrachotoxinin A 20α-2-ethyl-4-methyl-pyrrole-3-carboxylate.*

This assignment of structure was confirmed by the partial synthesis of batrachotoxin, viz., by acylation of the allylic 20α-hydroxyl of batrachotoxin with the mixed anhydride prepared from 2,4-dimethylpyrrole-

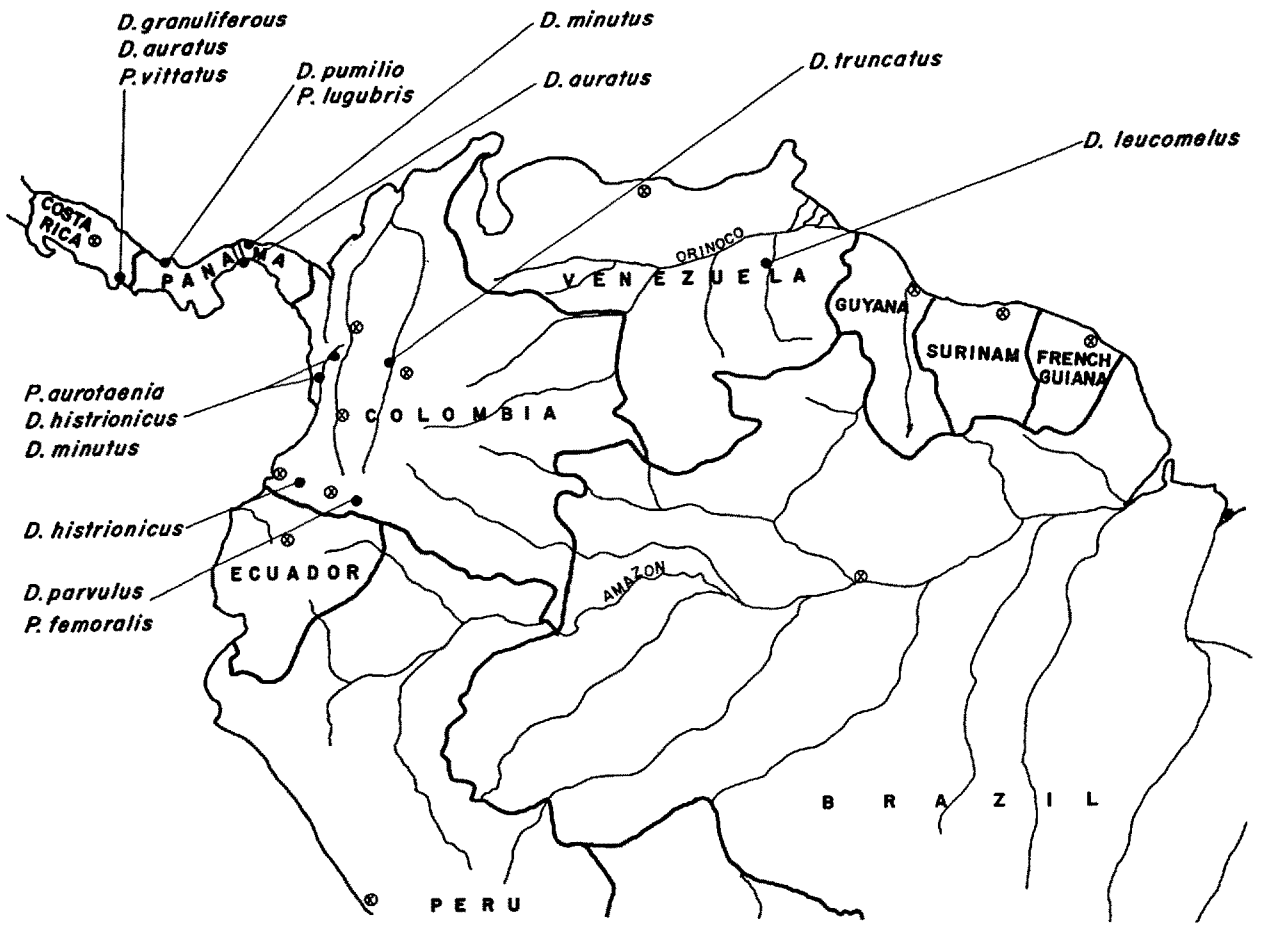


Fig. 4. Topography of the habitats of the various representatives of *Dendrobates* (D.) and *Phyllobates* (P.). Both *P. aurotaenia*, the kokoi or poison arrow frog, as well as *D. histrionicus* were collected in the Chocó region of Western Colombia along the banks of the river San Juan.

3-carboxylic acid and ethyl chloroformate (Figure 5), a method which fails with unactivated 20α-hydroxypregnanes. The synthetic material was identical in all respects with natural batrachotoxin.

Other synthetic analogs of batrachotoxin were prepared in a similar manner. The effect of pyrrole substitution on the toxicity of batrachotoxinin A is shown in Table IV.

Although ^{14}C-acetate, ^{14}C-serine and ^{14}C-mevalonate are readily incorporated into the cholesterol of the skin, neither pumiliotoxin (*Dendrobates pumilio*) nor batrachotoxin (*Phyllobates aurotaenia*) incorporate radioactivity from these or steroid precursors, e.g., ^{14}C-cholesterol, ^{14}C-pregnenolone or ^{14}C-progesterone[18].

The effects of batrachotoxin in a variety of systems can be explained, either as direct or indirect consequences of the depolarization of electrically excitable membranes. The mechanism of action of batrachotoxin in eliciting membrane depolarization, therefore, assumes prime importance. The hypothesis that *batrachotoxin causes a marked dose-dependent and irreversible increase in permeability of membranes to Na$^+$* is compatible with all of the observations at the present time. Indeed, the magnitude of the depolarization elicited by batrachotoxin in the giant squid axon (Figure 6)[19] and Purkinje fiber[20] can be explained only on the basis of an increase in membrane permeability to Na$^+$.

This interpretation is especially attractive in view of the fact that *tetrodotoxin is a specific antagonist of batrachotoxin*. Tetrodotoxin is known to interfere with generation of action potentials in nerve and muscle by blocking passive diffusion of sodium ions into the cells. Batrachotoxin is, as expected, less effective in media containing low concentrations of Na$^+$. The inhibitory effect of Ca^{++} on the action of batrachotoxin may be explained by membrane-stabilizing properties of this ion which antagonize permeation of membranes by other ions, such as Na$^+$. Such antagonism might also pertain if batrachotoxin, like the cardiac glycosides, blocked outflow of Na$^+$ by inhibition of the Na$^+$-K$^+$-activated ATPase. However, batrachotoxin does not inhibit this enzyme[21], nor does it decrease the short-circuit current in membranes as do the cardiac glycosides. The time course of events elicited by batrachotoxin in various preparations is increased by concomitant electrical stimulation. This is to be expected, since both batrachotoxin and electrical stimulation tend to increase membrane permeability to Na$^+$, so that their combined effect should be additive. Alternations in the repolarization phase of

Table IV. Effect of the ester moiety on the toxicity of batrachotoxinin A esters on s.c. administration in mice

20α-Ester moiety	LD_{50} (μg/kg)
None (batrachotoxinin A)	1000
2,4-Dimethylpyrrole-3-carboxylate (batrachotoxin)	2
2-Ethyl-4-methylpyrrole-3-carboxylate (homobatrachotoxin)	3
2,5-Dimethylpyrrole-3-carboxylate	2.5
4,5-Dimethylpyrrole-3-carboxylate	260
2,4,5-Trimethylpyrrole-3-carboxylate ('superbatrachotoxin')	1
2,4-Dimethyl-5-ethylpyrrole-3-carboxylate	8
2,4-Dimethyl-5-acetylpyrrole-3-carboxylate	250
N,2,4,5-Tetramethylpyrrole-3-carboxylate	>1000
Pyrrole-2-carboxylate	>1000

[18] D. F. Johnson and J. W. Daly, Biochem. Pharmac., in press (1971).

[19] T. Narahashi, E. X. Albuquerque and T. Deguchi, Nature, New Biology *229*, 222 (1971).

[20] P. M. Hogan and E. X. Albuquerque, J. Pharmac. exp. Ther. *176*, 529 (1971).

[21] F. C. Kauffman, E. X. Albuquerque, B. Witkop and J. W. Daly, Abstracts IV. Int. Pharmac. Congress, Basel 1969 (Karger, Basel 1970), p. 511.

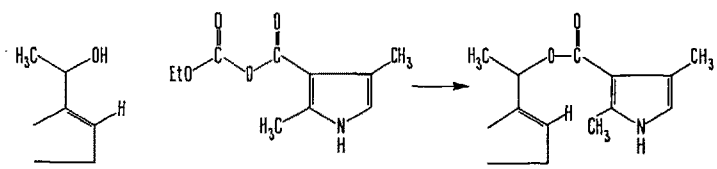


Fig. 5. Partial synthesis of batrachotoxin from batrachotoxinin A.

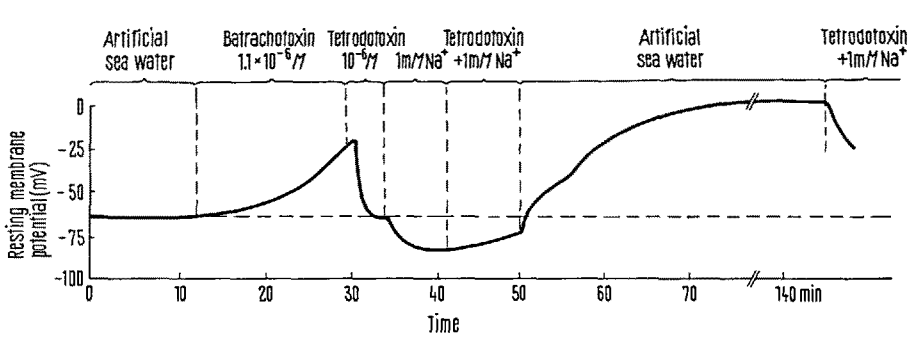


Fig. 6. Effect of batrachotoxin, tetrodotoxin and low concentrations of Na$^+$ on the resting membrane potential of an intact squid axon[19].

Table V. Response of various biological preparations to batrachotoxin and the effect of other parameters

Preparation	Response to batrachotoxin	Effect on response to batrachotoxin				
		Effect of electrical stimuli	Effect of low Na^+	Effect of high Ca^{++}	Effect of low Ca^{++} + EGTA	Effect of tetrodotoxin
Neuromuscular juncture (rat)	Block of muscle twitch in response first to A) indirect stimulation and then to B) direct stimulation	Accelerates A) and B)	Delays	Delays A)	–	–
	Transient increase in spontaneous transmitter release	–	–	Delays	Antagonizes	Prevents
	Muscle contracture, A) first transient phase, B) second prolonged phase	–	Antagonizes A), little effect on B)	Delays or prevents	Prevents	Prevents
	Depolarization of muscle fiber		Antagonizes	Delays	–	Prevents
	Block of action potential in muscle fiber	–	–	–	Little effect	–
	Damage to sarcotubular system	–	–	–	–	Prevents
Axon (squid)	Depolarization and resultant block of action potential	Accelerates	Prevents	–	–	Prevents
Intact heart, superior cervical ganglion (rabbit)	A) Alteration of action potential, followed by B) depolarization and block of ganglionic transmission	Accelerates B)	–	–	–	–
Heart Purkinje fiber (dog)	A) Alteration of action potential, and B) depolarization	–	Prevents B)	Delays B)	–	Prevents B)
Brain slice (guinea-pig)	Enhanced formation of cyclic AMP	–	Antagonizes	Antagonizes	Antagonizes	Prevents

action potentials are observed, as would be predicted, for electrogenic membranes with enhanced Na^+ permeability.

The secondary effects of batrachotoxin, such as increase in spontaneous transmitter release and muscle contracture in neuromuscular preparations and cyclic AMP formation in brain slices, appear to result from membrane depolarization. Thus, at a certain critical level of membrane depolarization, spontaneous transmitter release is greatly increased. Cessation of transmitter release may then reflect further depolarization of the presynaptic terminal past this critical level. The first phase of muscle contracture coincides with depolarization of the muscle membrane. The second sustained contracture may be due to disruption of the sarcotubular system with a concomitant release of its stores of Ca^{++}. This disruption could well be due to osmotic effects of elevated levels of intracellular Na^+. The enhanced formation of cyclic AMP in brain slices in the presence of batrachotoxin is postulated to be due to a depolarization-evoked increase in extracellular levels of adenosine[22].

Batrachotoxin irreversibly increases the permeability of membranes to Na^+. After treatment with batrachotoxin the membrane both in the muscle fiber and in the squid axon can be depolarized or repolarized by manipulation of Na^+ concentration or by addition and removal of tetrodotoxin, a reversible inhibitor (Figure 6).

Although the effect of batrachotoxin on membrane potential and permeability and the antagonistic action of tetrodotoxin, of reduced levels of Na^+ or of elevated levels of Ca^{++} finds parallels in all preparations investigated (Table V), the sensitivities of various membranes appears to differ widely. The heart Purkinje cell is extremely sensitive, the neuromuscular preparation less so and the squid axon least sensitive to batrachotoxin. This sensitivity to batrachotoxin is markedly reduced (temperature coefficient of about 2.8) at lower temperatures[23].

While we still have a poor understanding of the way in which familiar therapeutics, such as aspirin and morphine, exert their action, it took less than 3 years to define the basic mechanism of action of the novel pharmacon batrachotoxin. Its selective pharmacological activity in irreversibly increasing membrane permeability to Na^+ provides researchers with *a valuable tool for the study of electrogenic membranes*[24]. Current investigations with synthetic analogs and homologs of batrachotoxin have given promise of developing agents which at lower toxicity have altered activity toward different types of electrogenic membranes. Such agents might eventually find use as therapeutic agents. A situation which is being explored at the present time is muscular dystrophy, a syndrome in which sodium permeability and transmission of nerve impulses are both impaired[25].

[22] H. SHIMIZU, C. R. CREVELING and J. W. DALY, Proc. natn. Acad. Sci., USA *65*, 1053 (1970); Molec. Pharmac. *6*, 184 (1970).

[23] J. E. WARNICK, E. X. ALBUQUERQUE and F. M. SANSONE, J. Pharmac. exp. Ther. *176*, 497 (1971).

[24] E. X. ALBUQUERQUE, J. W. DALY and B. WITKOP, Science *172*, 995 (1971).

[25] E. X. ALBUQUERQUE and J. E. WARNICK, Science, *172*, 1260 (1971).

What is opened up by these batrachotoxin studies is the nature of the critical complex between sodium ions and the selectivity mechanism of the sodium-preferring and potassium-preferring channels in the surface of the nerve fibers. These ionic movements determine the characteristic shape and time course of the propagated nerve action potential. Unpublished observations of E. X. ALBUQUERQUE point to the importance of sulfhydryl groups of a membrane-specific acceptor protein for batrachotoxin: either disulfide interchange by pretreatment with dithiothreitol or complex formation with *p*-chloromercuribenzoate protect the membrane from the effect of batrachotoxin.

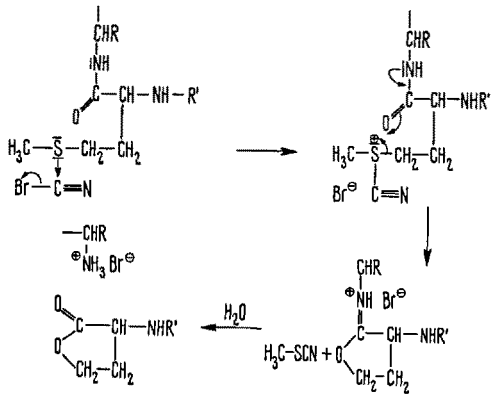


Fig. 7. Mechanism whereby cyanogen bromide cleaves peptide bonds next to methionine converting methionine to a C-terminal homoserine(lactone) and liberating a new peptide fragment.

D. *From neighboring group effects to the architecture of proteins and the catalytic activities of enzymes*

Neighboring group participation, intramolecular catalysis and *anchimeric assistance* by functional groups are designations intimately associated with the life work of the late SAUL WINSTEIN[26–28]. Participation of amino groups for the stepwise degradation of peptides are variations of this key theme[29,30]. The most useful application of this principle for the study of protein sequences is the cyanogen bromide cleavage (Figure 7)[31–34] which was first used to 'audit' the sequence of ribonuclease[35] arrived at by the 'accounting method' of overlapping tryptic peptides[36]. Errors in the 'accounting' method of proteins occur easily and are inevitable, as the 'cause celèbre' of the human growth hormone demonstrates[37]. In the past decade there has been no major large protein that yielded to 'sequencing'

[26] S. WINSTEIN and R. E. BUCKLES, J. Am. chem. Soc. *64*, 2780 (1942).
[27] W. LWOWSKI, Angew. Chemie *70*, 483 (1958).
[28] B. CAPON, Q. Rev. chem. Soc. *1964*, 45–111.
[29] L. A. COHEN and B. WITKOP, Angew. Chem. *73*, 253 (1961).
[30] K. L. KIRK and L. A. COHEN, J. org. Chem. *34*, 395 (1969).
[31] E. GROSS and B. WITKOP, J. Am. chem. Soc. *83*, 1510 (1961).
[32] E. GROSS, Meth. Enzymol. *11*, 238 (1967).
[33] B. WITKOP, Science *162*, 318 (1968).
[34] T. F. SPANDE and B. WITKOP, in *Handbook of Biochemistry* (Ed. H. A. SOBER; The Chemical Rubber Co., Cleveland, Ohio 1970), p. C138.
[35] E. GROSS and B. WITKOP, J. biol. Chem. *237*, 1856 (1962).
[36] B. WITKOP, Adv. Protein Chem. *16*, 294 (1961).
[37] H. D. NIALL, Nature, New Biology *230*, 90 (1971).

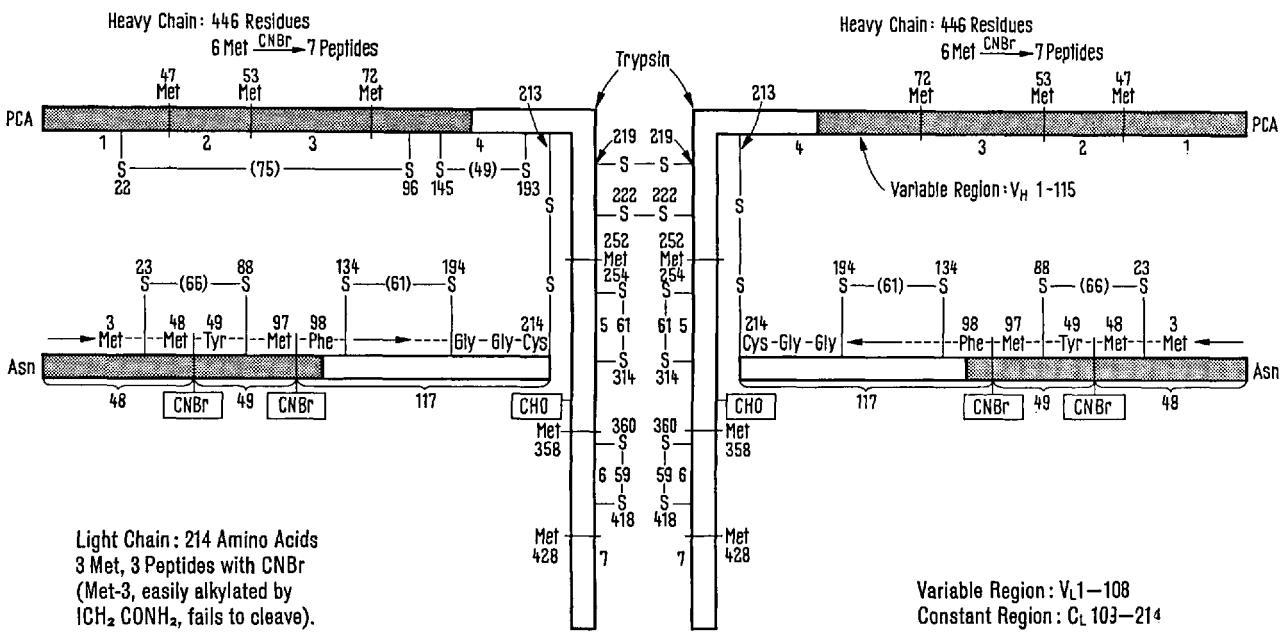


Fig. 8. The most useful application of the cyanogen bromide cleavage was in the antibody problem. This linear model of the 2 light and 2 heavy chains of the human γGl myeloma immunoglobulin Eu shows the variable regions as shaded areas and the location of the 3 and 6 methionine residues, respectively, and the 3 and 7 peptide fragments, respectively, resulting after cleavage[39,40]. Met-3 in the light chain is followed by Thr and does not cleave with CNBr. This failure of cleavage is often observed with Met-Ser or Met-Thr sequences (Table VI). CHO is carbohydrate and is probably not only attached to the heavy chain but also to the variable region of the light chain (V_L, shaded areas)[48].

Table VI. Cyanogen bromide cleavage of peptides and proteins

Peptide or protein	Molecular weight	Methionine residues	Reaction conditions Solvent	BrCN Met	Temperature (°C)	Time (h)	Reference
Tryptophan synthetase, fragment	2,000	1	0.1 *N* HCl	30	R.T.	24	[1]
Gastrin	2,000	2	Aqueous HF	excess	R.T.	24	[2]
Ribonuclease, bovine, S peptide	2,160	1	0.1 *N* HCl	30	R.T.	24	[3]
Aldolase, active site peptide, rabbit muscle	3,000	1	0.1 *N* HCl	excess	R.T.	24	[4]
Cholecystokinin-pancreozymin	3,500	3	0.1 *N* HCl	excess	21	24	[5]
Nisin	3,500	2	70% F		R.T.	24	[6]
Thyrocalcitonin, porcine	3,600	1	70% F	180	25	24	[7]
Thyrocalcitonin, bovine	3,600	1	70% F	50	25	24	[8]
Cytochrome-c 551, *pseudomonas*	8,000	2	0.1 *N* HCl	< 30	R.T.	24	[9]
Hormone, parathyroid	9,000	2	0.1 *N* HCl	excess	R.T.	24	[10]
Acyl carrier protein, *E. coli*	9,000	1	99% F	200	25	24	[11]
Ferredoxin, green alga	10,200	1	70% F	85	25	24	[12]
Histone, calf thymus (GAR)	10,600	1	–	–	–	–	[13,14]
Cytochrome-c	12,000	2	0.1 *N* HCl	excess	40	24	[15]
Cytochrome-c, baker's yeast	12,000	2	0.1 *N* HCl	excess	36	24	[16,17]
Cytochrome-c, horse-heart	12,000	2	–	–	–	–	[18–20]
Cytochrome-c, wheat germ	12,000	2	–	excess	–	–	[21]
Luteinizing hormone, CI-chain	12,500	4	70% F	300	R.T.	24	[22]
Cytochrome-c_2, *Rhodospirillum rubrum*	12,840	2	70% F	50	30	30	[23]
Thioredoxin	12,000	1	(HCl) (F_3CCOOH) 70% F	300	25	24	[24]
Ribonuclease, bovine pancreatic	13,700	4	0.1 *N* HCl	30	25	24	[25]
Ribonuclease cross-linked bovine pancreatic	13,700	4	0.1 *N* HCl	–	R.T.	24	[26]
Ribonuclease A, S-methylmethionine (29)	13,700	4	0.1 *N* HCl	30	R.T.	24	[27a]
S-carboxymethylmethionine (30)	13,700	4	0.05 *N* HCl	380	R.T.	24	[27b]
Phospholipase A (snake venom)	13,900	2	70% F	85	R.T.	24	[28]
Azurin	14,000	6	0.1 *N* HCl	excess	R.T.	24	[29]
Lysozyme, hen egg	14,400	2	70% F	excess	–	–	[30,31]
Lysozyme, bacteriophage λ	–	3 (one N-term)	0.1 *N* HCl	90–100	30	30	[32]
α-Lactalbumin	16,000	1	60% F	excess	R.T.	–	[33,34]
Nuclease, extracellular *Staph. aureus*	16,500–17,000	4	70% F	25–30	25	20	[35–37]
Hemoglobin V (lamprey)	16,600	4	70% F	60	R.T.	4.5	[38]
Myoglobin	18,000	2	0.1 *N* HCl	40	R.T.	24	[39]
Encephalitogenic basic protein from myelin	18,200	2	70% F	100	R.T.	24	[40]
Growth hormone, human	21,500	3	70% F	excess	R.T.	–	[41]
Growth hormone, bovine	20,800	3	70% F	excess	25	24	[42,43]
Growth hormone, porcine	22,000	3	70% F	70	R.T.	6	[44]

[1] GUEST and YANOFSKY, J. biol. Chem. *241*, 1 (1966).
[2] GREGORY, HARDY, JONES, KENNER and SHEPPARD, Nature, Lond. *204*, 931 (1964).
[3] GROSS and WITKOP, Biochemistry *6*, 745 (1967).
[4] C. Y. LAI, P. HOFFEE and B. L. HORECKER, personal communication (1969).
[5] MUTT and JORPES, Biochem. Biophys. Res. Commun. *26*, 392 (1967).
[6] GROSS and MORELL, J. Am. chem. Soc. *92*, 2919 (1970) and in press.
[7] POTTS JR., NIALL, KEUTMANN, BREWER JR. and DEBOTS, Proc. natn. Acad. Sci., USA *59*, 1321 (1968). – BARG JR., ENGLERT, DAVIS, COLUCCI, SNEDEKER, DZIOBKOWSKI and BELL, Biochemistry *9*, 1671 (1970).
[8] BREWER JR. and ROMAN, Proc. natn. Acad. Sci., USA *63*, 940 (1969).
[9] AMBLER, Biochem. J. *96*, 32P (1965).
[10] POTTS JR. and AURBACH, in *The Parathyroid Glands: Ultrastructure, Secretion and Function* (Eds. GAILLARD, TALMADGE and BUDY; University of Chicago Press, Chicago 1965).
[11] VANAMAN, WAKIL and HILL, J. biol. Chem. *243*, 6409 (1968).
[12] SUGENO and MATSUBARA, J. biol. Chem. *244*, 2979 (1969).
[13] SAUTIERE, STARBUCK and BUSCH, Fedn Proc. *27*, 777 (1968).
[14] DELANGE and FAMBROUGH, Fedn Proc. *27*, 392 (1968).
[15] CHU and YASUNOBU, Biochim. biophys. Acta *89*, 148 (1964).
[16] NARITA, TITANI, YAOI and MURAKAMI, Biochim. biophys. Acta *77*, 688 (1963).
[17] TITANI and NARITA, J. Biochem., Tokyo *56*, 241 (1964).
[18] BLACJ and LEAF, Biochemistry *96*, 693 (1964).
[19] CORRADIN and HARBURY, Biochim. biophys. Acta *221*, 489 (1970).
[20] MCGOWAN and STELLWAGEN, Biochemistry *9*, 3047 (1970).
[21] STEVENS, GLAZER and SMITH, J. biol. Chem. *242*, 2764 (1967).
[22] PIERCE, LIAO, CARLSEN and REIMO, J. biol. Chem. *246*, 866 (1971).
[23] DUS, SLETTEN and KAMEN, J. biol. Chem. *243*, 5507 (1968).
[24] HOLMGREN and REICHARD, Eur. J. Biochem. *2*, 187 (1967).
[25] GROSS and WITKOP, J. biol. Chem. *237*, 1856 (1962).
[26] MARFEY, UZIEL and LITTLE, J. biol. Chem. *240*, 3270 (1965).
[27a] LINK and STARK, J. biol. Chem. *243*, 1082 (1968).
[27b] GOREN and BARNARD, Biochemistry *9*, 974 (1970).
[28] SAMEJIMA, IWANAGA, SUZUKI and KAWANCHI, Biochim. biophys. Acta *221*, 417 (1970).
[29] AMBLER and BROWN, J. molec. Biol. *9*, 825 (1964).
[30] BONAVIDA, MILLER and SERCARZ, Fedn Proc. *26*, 339 (1967); Biochemistry *8*, 968 (1969).
[31] YOUNG and LEUNG, Biochemistry *9*, 2755 (1970).
[32] BLACK and HOGNESS, J. biol. Chem. *244*, 1982 (1969).
[33] BREW, VANAMAN and HILL, J. biol. Chem. *242*, 3747 (1967).
[34] BREW and HILL, J. biol. Chem. *245*, 4559 (1970).
[35] TANIUCHI and ANFINSEN, J. biol. Chem. *241*, 4366 (1966).
[36] TANIUCHI, ANFINSEN and SODJA, J. biol. Chem. *242*, 4736 (1967).
[37] OMENN, ONTJES and ANFINSEN, Biochemistry *9*, 313 (1970).
[38] LI and RIGGS, J. biol. Chem. *245*, 6149 (1970).
[39] EDMUNDSON, Nature, Lond. *198*, 354 (1963).
[40] CHAO and EINSTEIN, J. biol. Chem. *245*, 6397 (1970).
[41] LI, LIU and DIXON, J. Am. chem. Soc. *88*, 2050 (1966).
[42] FELLOWS JR., NUTTING, ROGOL and KOSTYO, Fedn Proc. *27*, 434 (1968).
[43] FELLOWS JR. and ROGOL, J. biol. Chem. *244*, 1567 (1969).
[44] MILLS, HOWARD, SCAPA and WILHELM, J. biol. Chem. *245*, 3407 (1970).

Continuation of Table VI.

Peptide or protein	Molecular weight	Methionine residues	Reaction conditions Solvent	BrCN Met	Temperature (°C)	Time (h)	Reference
Trypsinogen, bovine pancreatic	24,000	2	0.2 *N* HCl	40	30	30	[45]
Chymotrypsin	25,000	2	0.1 *N* HCl	< 30	R.T.	24	[46]
Thyrotropin (bovine) (carboxymethylated α and β subunits)	25,000	4 in each subunit	70% F	300	R.T.	24	[47]
Enterotoxin (staphylococcal)	28,500	8 (Two Met-Met!)	70% F	70	R.T.	24	[48]
Tryptophan synthetase A (*E. coli*)	28,800	5 (one N-term)	75% TFA	110	R.T.	30	[49]
Carbonic anhydrase B	30,000	2	70% F	200	25	24	[50]
Yeast enolase	33,600	4	70% F	30–3000	R.T.	1–6 days	[51]
Carboxypeptidase A	34,600	3	70% HF	excess	R.T.	24	[52–54]
S-Sulfopepsin	35,000	4	80% F	60	37	20	[55]
Actin (rabbit skeletal)	45,000–47,000	16–17	70% F	50	R.T.	20–24	[56a]
			70% F	130	25	16	[56b]
Streptokinase, *Streptococcus*	48,000	4	70% F	250	23	24	[57]
Kininogen, human	50,000	2	0.25 *N* HCl	5	35	20	[58]
Avidin (4 subunits)	70,000	2 per subunit	70% F	80	R.T.	20–2	[59]
Ovotransferrin	76,600	8	70% F	100	R.T.	5	[60]
Collagen	100,000	6–8	0.1 *N* HCl	100	30	15	[61,62]
Collagen, rat skin α 1-chain	93,000	7	0.1 *N* HCl	100	30	4	[63] (cf.[68–75])
α 2-chain	95,000	5	0.1 *N* HOAc	200	30	4	[64]
Collagen, chick skin α 1-chain	95,000	9	0.1 *N* HCl	100	30	4	[65]
Collagen, chick bone α 1-chain	95,000	9	0.1 *N* HCl	150	30	4	[66]
α 2-chain	95,000	5	0.1 *N* HCl	150	30	4	[67]
Myosin fragment (rabbit muscle)	100,000	ca. 28	70% F	500	25	18	[76]
β-Galactosidase, *E. coli*	135,000	24	70% F	50	R.T.	16–20	[77]
Spectrin (red cell membrane)	140,000–150,000	22	70% F	2.5	R.T.	24	[78]
Immunoglobulin IgG, rabbit partial cleavage, active fragment	~150,000	10	0.3 *N* HCl	200	R.T.	4	[79]
Complete cleavage, inactive fragment	~150,000	10	70% F	excess	R.T.	24	
Immunoglobulin IgG, human	150,000	20	70% F	excess	R.T.	4	[80–85]
Aldolase, rabbit muscle (4 units of 40,000)	160,000	4 × 3	70% F	30	R.T.	22	[4]
Myosin A	500,000	130	0.1 *N* HCl	> 50	25	22	[86]
Glutamine synthetase (sheep brain)	500,000	119	0.1 *N* HCl	30	25	24	[87]
Thyroglobulin, native (2 subunits of 330,000)	660,000	46	70% F	150	25	20	[88]

[45] Hofmann, Biochemistry *3*, 356 (1965).
[46] Koshland jr., Strumeyer and Ray jr., Brookhaven Symp. Biol. *15*, 101 (1962).
[47] Shome, Liao, Howard and Pierce, J. biol. Chem. *246*, 833 (1971).
[48] Huang and Bergdoll, J. biol. Chem. *245*, 3518 (1970).
[49] Drapeau and Yanofsky, J. biol. Chem. *242*, 5434 (1967).
[50] Marrio, Gulian and Laurent, Biochim. biophys. Acta *221*, 662 (1970).
[51] Brewer, Fairwell, Travis and Lovins, Biochemistry *9*, 1011 (1970).
[52] Bargetzi, Thompson, Sampath Kumar, Walsh and Neurath, J. Biol. Chem. *239*, 3767 (1964).
[53] Nomoto, Srinivasan, Bradshaw, Wade and Neurath, Biochemistry *8*, 2755 (1969).
[54] Pétra and Neurath, Biochemistry *8*, 2466 (1969).
[55] Kostka, Moravek, Kluh and Keil, Biochim. biophys. Acta *175*, 459 (1969).
[56a] Adelstein and Kuehl, Biochemistry *9*, 1355 (1970).
[56b] Elzinga, Biochemistry *9*, 1365 (1970).
[57] Morgan and Henschen, Biochim. biophys. Acta *181*, 93 (1969).
[58] Axen, Gross, Witkop, Pierce and Webster, Biochem. Biophys. Res. Commun. *23*, 92 (1966).
[59] DeLange, J. biol. Chem. *245*, 9071 (1970).
[60] Phillips and Azari, Biochemistry *10*, 1160 (1971).
[61] Bornstein and Piez, Biochemistry *5*, 3460 (1966).
[62] Nordwig and Dick, Biochim. Biophys. Acta *97*, 179 (1965).
[63] Butler, Piez and Bornstein, Biochemistry *6*, 3771 (1967).
[64] Fietzek and Piez, Biochemistry *8*, 2129 (1969).
[65] Kang, Piez and Gross, Biochemistry *8*, 1506 (1969).
[66] Miller, Lane and Piez, Biochemistry *8*, 30 (1969).
[67] Lane and Miller, Biochemistry *8*, 2134 (1969).
[68] Deshmukh and Nimni, Biochemistry *10*, 1640 (1971).
[69] Kang, Piez and Gross, Biochemistry *8*, 3648 (1969).
[70] Kang, Igarashi and Gross, Biochemistry *8*, 3200 (1969).
[71] Bornstein, Biochemistry *8*, 63 (1969).
[72] Click and Bornstein, Biochemistry *9*, 4699 (1970).
[73] Epstein jr., Scott, Miller and Piez, J. biol. Chem. *246*, 1718 (1971).
[74] Miller and Matukas, Proc. natn. Acad. Sci., USA *64*, 1264 (1969).
[75] Miller, Biochemistry *10*, 1652 (1971).
[76] Huszar and Elzinga, Biochemistry *10*, 229 (1971).
[77] Steers jr., Craven, Anfinsen and Bethune, J. biol. Chem. *240*, 2478 (1969).
[78] Marchesi, Steers, Marchesi and Tillack, Biochemistry *9*, 50 (1970).
[79] Cahnmann, Arnon and Sela, J. biol. Chem. *240*, PC 2762 (1965).
[80] Waxdal, Konigsberg and Edelman, Biochemistry 7, 1967 (1968).
[81] Yoo and Pressman, Fedn Proc. *27*, 683 (1968).
[82] Press, Piggott and Porter, Biochem. J. *99*, 356 (1966).
[83] Wikler, Köhler, Sinoda and Putnam, Fedn Proc. *27*, 559 (1968).
[84] Edelman, Cunningham, Gall, Gottlieb, Rutishauser and Waxdal, Proc. natn. Acad. Sci., USA *63*, 78 (1969).
[85] Pitcher and Konigsberg, J. biol. Chem. *245*, 1267 (1970).
[86] Young, Blanchard and Brown, Proc. natn. Acad. Sci., USA *61*, 1087 (1968).
[87] Ronzio, Rowe, Wilk and Meister, Biochemistry *8*, 2670 (1969).
[88] Nissley, Cittanova and Edelhoch, Biochemistry *8*, 443 (1969).

without the benefit of the cyanogen bromide cleavage[38]. Table VI, in order of rising molecular weight and complexity, lists the peptides and proteins whose sequences have been determined with the help of this method.

I must admit that at times it was not easy to resist the temptation to get lost in applications of this selective chemical cleavage to many proteins. However, after an initial lag period of several years more expert protein specialists took up the new method and achieved results as spectacular as the complete architecture of γ-globulin (Figure 8)[39], a protein molecule 25 times as large as insulin and containing 19,996 atoms[40]. Knowledge of this most vital protein has opened up the vast fields of cellular differentiation, antibody genes, recognition patterns, immune tolerance, organ transplantation, autoimmune diseases[41] and variations in the primary structure of antibodies during the course of immunization[42]. 'Forbidden antibodies' may play a role in nervous disorders, such as *myasthenia gravis*.

Another almost equally significant protein is collagen which makes up the connective tissue and is the major protein constituent of vertebrates and invertebrates. Its two different kinds of α-chains contain each about 1000 amino acids, therefore the linear order of about 2000 amino acids must be determined. There are, however, certain collagen species with 3 identical α-chains, a fact which can be established by examination of the cyanogen bromide peptides[43]. There are only 5–9 residues of methionine in each α-chain (Figure 9). Therefore the cyanogen bromide cleavage has been the method of choice for the establishment of the primary structure of collagen[44].

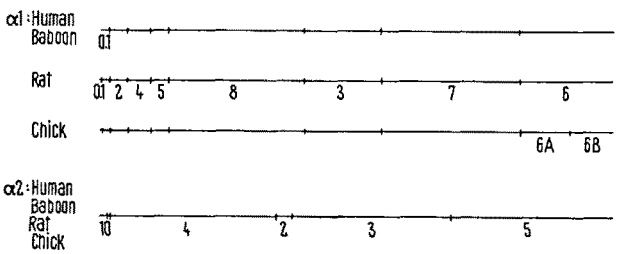


Fig. 9. The distribution of methionine in the $\alpha 1$ and $\alpha 2$ chains of collagens from several species as determined by characterization of the cyanogen bromide peptides. The vertical lines show the positions of the methionyl residues; the distances between them are proportional to the size of the peptides. The collagens are from skin (human, baboon, rat and chick), tendon (rat) and bone (chick)[46].

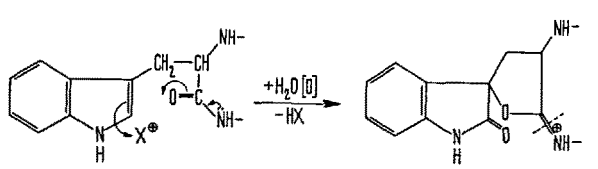


Fig. 10. Neighboring group effects in the cleavage of tryptophan through spirodioxindole-iminolactones.

The macromolecular assembly of the triple-stranded tropocollagen units is dependent on intra- and intermolecular cross links. Again cyanogen bromide cleavage has shown that lysyl residues located near the N-terminal region are converted into the δ-semialdehyde of α-aminoadipic acid and that this step is followed by an aldol condensation, yielding an α,β-unsaturated aldehyde which serves as the intramolecular cross link[45,46]. These aldehydes have now been located along the whole length of the collagen molecule, again with the help of the cyanogen bromide cleavage[47]. The formation of these cross linkages may be subject to hormonal control (corticosteroids), and may increase with age or decrease in rheumatoid disorders. In the latter case fragments of collagen, formed by lysosomal enzymes in the process of inflammation, may act as antigens so that some disorders, such as polyarthritis, appear as autoimmune diseases and respond to immunosuppressants. The source for such 'autoantigens', especially in rheumatoid or polyarthritis characterized by the pathological deposition of *amyloid protein*, a unique glyco-protein (MW 7200), (amyloidosis) in tissues – may also be the variable region of the light chain of immunoglobulin (Figure 8)[48].

Related to these important etiological and clinical questions is the role that proline hydroxylase plays, the enzyme that selectively and stereospecifically[49] converts the proline-containing polypeptide precursor ('procollagen') into collagen. This hydroxylase appears to be involved in wound healing, tissue regeneration, in rheumatoid arthritis and in several clinical syndromes[50], such as Paget's disease, acromegaly[51] or hepatoma[52]. Its manipulation by either stimulation or inhibition in general and in synovial fluids in particular, is a matter of practical interest.

[38] T. F. SPANDE, B. WITKOP, Y. DEGANI and A. PATCHORNIK, Adv. Protein Chem. *24*, 205 (1970).
[39] G. M. EDELMAN and W. E. GALL, A. Rev. Biochem. *38*, 415 (1969).
[40] G. M. EDELMAN, Scient. American *223*, 34 (1970).
[41] J. A. GALLY and G. M. EDELMAN, Nature, Lond. *227*, 341 (1970).
[42] J.-C. JATON, M. D. WATERFIELD, M. N. MARGOLIES, K. J. BLOCH and E. HABER, Biochemistry *10*, 1583 (1971).
[43] E. J. MILLER, Biochemistry *10*, 1652 (1971).
[44] E. H. EPSTEIN, R. D. SCOTT, E. J. MILLER and K. A. PIEZ, J. biol. Chem., in press (1971).
[45] K. A. PIEZ, A. Rev. Biochem. *37*, 547 (1968).
[46] W. TRAUB and K. A. PIEZ, Adv. Protein Chem. *25* (in press) (1971).
[47] K. DESHMUKH and M. E. NIMNI, Biochemistry *10*, 1640 (1971).
[48] G. G. GLENNER, J. HARBAUGH, J. I. OHMS, M. HARADA and P. CUATRECASAS, Biochem. Biophys. Res. Commun. *41*, 1287 (1970) Science, *172*, 1150 (1971).
[49] Y. FUJITA, A. GOTTLIEB, B. PETERKOFSKY, S. UDENFRIEND and B. WITKOP, J. Am. chem. Soc. *86*, 4709 (1964).
[50] Second European Symposium on Connective Tissue Research, Hannover (Germany), September 1970, in Z. klin. Chem. klin. Biochem. *9*, 59 (1971).
[51] A. SJOERDSMA, S. UDENFRIEND, H. KEISER and E. C. LEROY, Ann. intern. Med. *63*, 672 (1965).
[52] N. E. ROBERTS and S. UDENFRIEND, J. natn. Cancer Inst. *45*, 277 (1970).

After this excursion into proteins we may again return to the basis of the cyanogen bromide cleavage, the neighboring group effect, and examine its role in enzymatic catalysis. Here it is important to realize that what in ordinary hydrolysis of a substrate by acid or base is a *slow intermolecular reaction* by contrast becomes a *fast intramolecular process* in the enzyme-substrate complex. An enzyme does not simply bind its substrate, but 'freezes' it by various types of interaction and at various points of contact. Analogously, when an intramolecular system is further modified by severe freezing of rotational motion, a large fraction of the molecules are forced to exist in a single conformation or 'population'. L. A. COHEN and his colleagues, in the Laboratory of Chemistry, have utilized this principle of 'stereopopulation control' to produce ground states so similar in conformation to transition states that rate enhancement factors as great as 10^{11}, or 10^{16} compared with the *inter*molecular process, have been attained[53]. In addition to providing reaction rates approaching those of enzymes, the technique of 'stereopopulation control' provides the most satisfying general model of an enzyme-substrate complex to date, integrating into a single model, concepts such as propinquity, orientation, 'orbital steering', unprofitable rotamer distribution, non-bonded repulsion, interorbital penetration, solvent exclusion, and extended residence time[54].

E. *Selective attenuation of toxins*

N-Bromosuccinimide (NBS) can either cleave tryptophan peptides by another type of neighboring group effect (Figure 10) or merely convert the indole moiety to oxindole or dioxindole[55]. The buffer system, the nature of the protein and other subtle factors determine the course of the reaction[56].

The antigenic I-peptide of tobacco mosaic virus (TMV), which contains one single tryptophan in a chain of 41 amino acids, can be carefully oxidized with NBS without cleavage or loss of antigenicity[57]. Likewise, cobratoxin, the venom of the cobra, a linear peptide of 62 amino acids, contains a single tryptophan which can be selectively modified by ozone or by NBS[58]. In this case toxicity is lost completely with full retention of antigenicity[59]. Such observations raise the possibility of using NBS for the attenuation of toxins for the preparation of toxoids for immunization. At the present time relatively unspecific reagents, such as formaldehyde or β-propiolactone, are used for this purpose.

F. *From 'False Transmitters' to 'Chemical Sympathectomy'*

In 1959 my first collaborator from Japan, Dr. SIRO SENOH, began a study on non-enzymatic conversions of dopamine to norepinephrine and trihydroxyphenethylamines. What started out as variations on a theme of the Thiele addition, viz. 1,2; 1,4 and 1,6-addition to dopamine-quinone (Figure 11), ended more than a decade later as one of the most interesting developments in the field of catecholamines.

This SENOH-WITKOP amine (6-hydroxydopamine or 2,4,5-trihydroxyphenethylamine) in low concentrations serves as a 'false transmitter'[60], i.e. as an amine mimicking norepinephrine (NE), it replaces and releases NE from storage sites, e.g. in mouse heart[61]. In higher concentrations this amine first functionally impairs nerve transmission and then produces degeneration of the nerve terminal. We have shown that 6-hydroxydopamine has a very low oxidation potential and easily forms aminochromes[62]. This instability makes the detection of the SENOH-WITKOP amine as a labile metabolite of dopamine very difficult[63].

The SENOH-WITKOP amine has been invoked in a hypothesis on the possible etiology of schizophrenia, in which reduced activity of dopamine β-hydroxylase results in release and autoxidation of dopamine. The resulting damage to adrenergic terminals supposedly leads to schizophrenia, manic depression or Parkinsonism[64]. So far this hypothesis has not found much acceptance. The product of enzymatic *m-O*-methylation by catechol *O*-methyltransferase (COMT), 2,4-dihydroxy-5-methoxyphenethylamine[65] as well as the corresponding 6-hydroxynormetanephrine analog[66] are much more stable and are potential metabolites, isomeric with metabolites of mescaline (by demethylation)[67] or of the false transmitter 3,4,5-trihydroxyphenethylamine[68] by remethylation.

By the use of electron microscopy and tritiated substrates, H. THOENEN and his collaborators have shown that 6-hydroxydopamine, in a largely selective process,

[53] S. MILSTIEN and L. A. COHEN, Proc. natn. Acad. Sci., USA *67*, 1143 (1970).
[54] T. C. BRUICE, A. BROWN and D. O. HARRIS, Proc. natn. Acad. Sci., USA *68*, 658 (1971). – D. R. STORM and D. E. KOSHLAND JR., *66*, 445 (1970). – J. REUBEN, *68*, 563 (1971).
[55] N. M. GREEN and B. WITKOP, Ann. N.Y. Acad. Sci. *26*, 659 (1964).
[56] T. F. SPANDE and B. WITKOP, Methods Enzym. *11*, 498 (1967).
[57] B. WITKOP, Adv. Protein. Chem. *16*, 289 (1961).
[58] C. C. CHANG and K. HAYASHI, Biochem. Biophys. Res. Commun. *37*, 841 (1969).
[59] A. T. TU, B. HONG and T. N. SOLIE, Biochemistry *10*, 1295 (1971).
[60] J. J. KOPIN, R. A. COHEN, I. J. KOPIN, C. R. CREVELING, J. M. MUSACCHIO, J. E. FISCHER, J. R. CROUT and J. R. GILL JR., cf. Ann. intern. Med. *65*, 347 (1966).
[61] J. W. DALY, C. R. CREVELING and B. WITKOP, J. med. Chem. *9*, 273, 280, 284 (1966).
[62] S. SENOH and B. WITKOP, J. Am. chem. Soc. *81*, 6231 (1959).
[63] S. SENOH, B. WITKOP, C. R. CREVELING and S. UDENFRIEND, J. Am. chem. Soc. *81*, 1768 (1959).
[64] L. STEIN and C. WISE, Science *171*, 1032 (1971).
[65] J. DALY, L. HORNER and B. WITKOP, J. Am. chem. Soc. *83*, 4787 (1961).
[66] J. W. DALY, J. BENIGNI, R. MINNIS, Y. KANAOKA and B. WITKOP, Biochemistry *4*, 2513 (1965).
[67] J. W. DALY, J. AXELROD and B. WITKOP, Ann. N.Y. Acad. Sci. *96*, 37 (1961).
[68] C. R. CREVELING, J. W. DALY and B. WITKOP, J. med. Chem. *11*, 595 (1968).

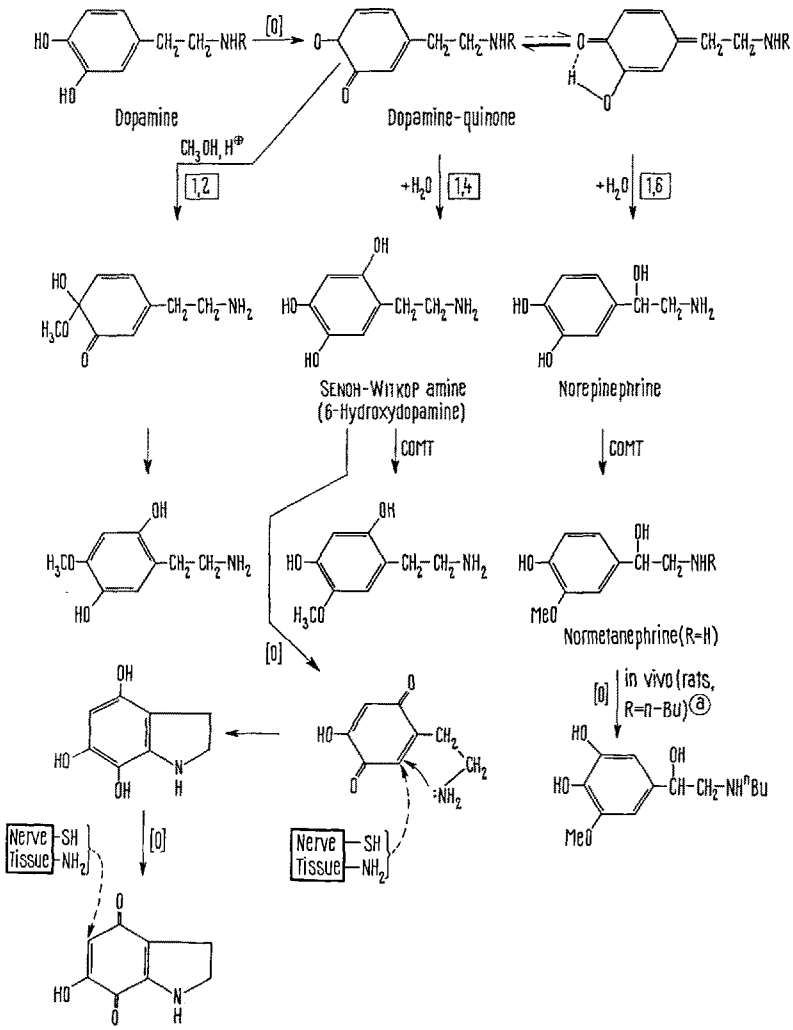


Fig. 11. 1,2,- 1,4- and 1,6-additions of nucleophiles (H_2O, CH_3OH, etc.) to (N-acylated)dopamine-quinone.
ⓐ The in vivo formation of 1-(3-methoxy-4,5-dihydroxyphenyl)-1-hydroxy-2-butylaminoethane from N-butylnormetanephrine, a metabolite of Bamethane® [P. B. DANNEBERG, R. HAHN and A. MENTRUP, personal communication] is analogous to the 5-hydroxylation of 3-methoxytyramine [F. BENINGTON and R. D. MORIN, Experientia *24*, 33 (1968)].

destroys adrenergic neurons and that this *chemical sympathectomy* provides a new and valuable tool in the investigation of the physiology and pharmacology of peripheral and central adrenergic neurons[69,70]. These findings have led to the First International Symposium on 6-Hydroxydopamine[71].

While 6-hydroxydopamine is unable to penetrate the blood-brain barrier, in contrast to α-amino acids capable of ATP-mediated *active* transport or lipophilic derivatives of catecholamines[72], the corresponding amino acid, 6-hydroxydopa, presumably in its L-form, crosses into the brain, undergoes decarboxylation and causes peripheral and central release and depletion of norepinephrine[73].

As to the mechanism of selective damage to adrenergic neurons, 6-hydroxydopamine, by virtue of its similarity to norepinephrine, is transported into the neuron where it probably undergoes oxidation to the hydroxyquinone which then acts like other polyhydric phenolic tanning agents and reacts with nucleophilic groups, presumably from the 'receptor protein'[74].

Other amines which have purely depleting ability, such as 3,5-dihydroxy-4-methoxyphenethylamine[75] and the corresponding amino acid, 3,5-dihydroxy-4-methoxyphenylalanine[76,77] were highly active and better hypotensives in genetically hypertensive rats[77] than α-methyldopa but failed in the clinic, probably because of poor resorption.

[69] H. THOENEN, J. P. TRANZER and G. HÄUSLER, in *New Aspects of Storage and Release Mechanisms of Catecholamines* (Springer Verlag, Berlin, Heidelberg, New York 1970).
[70] H. THOENEN, *Perspectives in Neuropharmacology*, published in honor of the 60th birthday of J. AXELROD, by Oxford University Press, 1971.
[71] North Holland Publishers, Amsterdam 1971, in press.
[72] C. R. CREVELING, J. W. DALY, T. TOKUYAMA and B. WITKOP, Experientia *25*, 26 (1969).
[73] H. H. ONG, C. R. CREVELING and J. W. DALY, J. med. Chem. *12*, 458 (1969). – B. A. BERKOWITZ, S. SPECTOR, A. BROSSI, A. FOCELLA and S. TEITEL, Experientia *26*, 982 (1970).
[74] W. J. SMITH and N. KIRSHNER, Molec. Pharmac. *3*, 52 (1967).
[75] C. R. CREVELING, J. W. DALY and B. WITKOP, J. Pharmac. exp. Ther. *158*, 46 (1967).
[76] H. THOENEN, W. HAEFELY, G. HAEUSLER and A. HÜRLIMANN, J. Pharmac. exp. Ther. *162*, 70 (1968).
[77] S. SPECTOR, R. TABEI, C. R. CREVELING, J. W. DALY, B. WITKOP and A. SJOERDSMA, Life Sci. *7*, 943 (1968).

G. *Catechol O-methyltransferase and the Adrenergic Receptor*

The discovery which brought fame and the Nobel prize to JULIUS AXELROD, was the realization that norepinephrine is inactivated in metabolism by enzymatic *m-O*-methylation to normetanephrine[78], a compound we had synthesized in anticipation of this pathway[79]. We then studied the dynamic aspects of enzymatic methylation and demethylation in vitro and in vivo[80] because *p-O*-methylation was thought to be a possible process in schizophrenics[81] leading to the catatonogenic 'metabolite' 3,4-dimethoxyphenethylamine[82], the so-called 'pink spot' which members of my laboratory have identified as of dietary origin[83]. Purification and characterization of COMT are being actively and successfully pursued by my colleagues[84]. The 100% pure enzyme still contains the carbohydrates, galactose, mannose and possibly glucose which originally must have been linked to the membrane or receptor site.

The manipulation of this enzyme which may be part of, or very close to, the adrenergic receptor site, especially its inhibition, is a matter of practical interest. The first successful competitive inhibitors were pyrogallol, tropolone, 3,4-dihydroxyphenylacetamide[85] and 3,5-dihydroxy-4-methoxyphenyl derivatives[86]. More recently we have made use of the thiol group, presumably present at or near the active site of COMT, and have found that mercapto analogs of catecholamines irreversibly bind to, and inhibit, COMT in vitro[87]. This approach should be applicable to other SH-dependent enzymes.

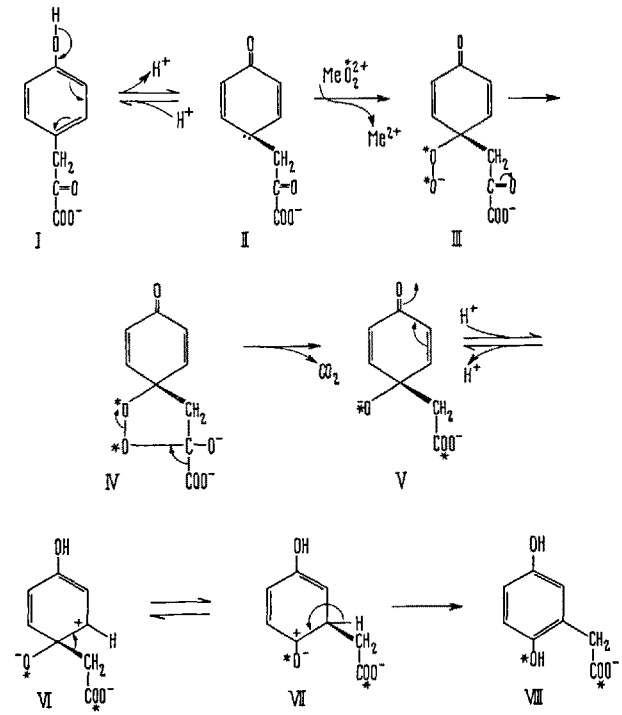


Fig. 12

[78] J. AXELROD, S. SENOH and B. WITKOP, J. biol. Chem. *233*, 697 (1958).

[79] J. AXELROD, Nobel Lecture, 1970, *Les Prix Nobel* (Imprimerieal Royal P. A. Norstedt & Söner, Stockholm 1971, p. 189.

[80] J. W. DALY, J. AXELROD and B. WITKOP, J. biol. Chem. *235*, 1155 (1960).

[81] F. A. KUEHL, in *Amines and Schizophrenia* (Eds. H. E. HIMWICH, S. S. KETY and J. R. SMYTHIES; Pergamon Press, Oxford, London 1967), p. 23.

[82] A. J. FRIEDHOFF and E. VAN WINKLE, in *Amines and Schizophrenia* (Eds. H. E. HIMWICH, S. S. KETY and J. R. SMYTHIES; Pergamon Press, Oxford, London 1967), p. 19.

[83] J. R. STABENAU, C. R. CREVELING and J. W. DALY, Am. J. Psychiat. *127*, 611 (1970).

[84] C. R. CREVELING, N. DALGARD, H. SHIMIZU and J. W. DALY, Molec. Pharmac. *6*, 691 (1970).

[85] G. BARTHOLINI, I. KURIMA and A. PLETSCHER, Nature, Lond. *230*, 533 (1971).

[86] B. NIKODIJEVIC, J. DALY and C. R. CREVELING, J. Pharmac. exp. Ther. *174*, 83 (1970).

[87] W. B. LUTZ, C. R. CREVELING, J. W. DALY and B. WITKOP, unpublished.

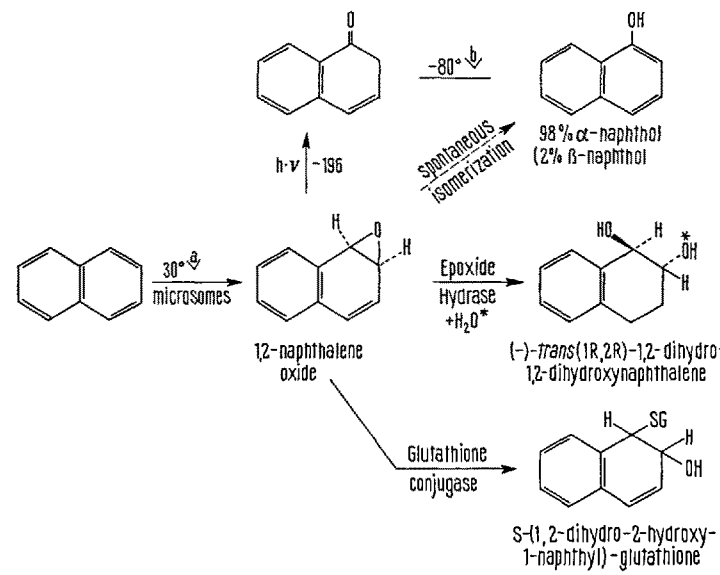


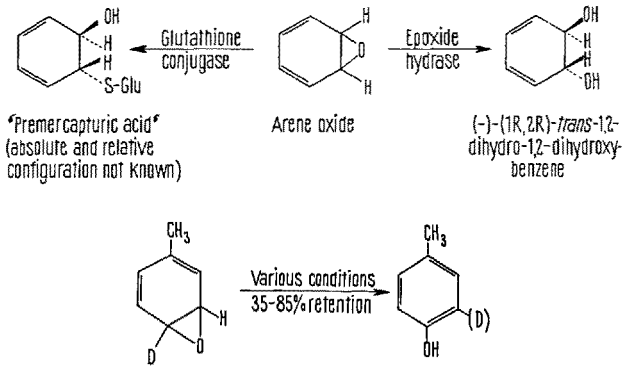
Fig. 13. Biochemical and photochemical reactions of 1,2-naphthalene oxide. a) Rate-limiting step, no isotope effect. b) Non-ratelimiting step, involving isotope effect.

H. *The labile metabolite: From arene oxides and the NIH shift to long-range toxicity of aromatic drugs and cancerogenic hydrocarbons*

My personal interest in labile metabolites started with the synthesis of formylkynurenine by ozonization of tryptophan[88] and by the synthesis of 5- and 7-hydroxytryptophans[89], of which only the former is a substrate for aromatic decarboxylase[90]. There is still room for even more labile hydroxylated metabolites of serotonin or melatonin[91]. There was no reason to suspect at that time that the hydroxylation of tryptophan to 5-hydroxytryptophan by soluble hydroxylases, or of tryptamine to 6-hydroxytryptamine by particle-bound microsomal hydroxylases, involved a shift of hydrogen from position 5→4 or from position 6→7, respectively[92]. The rearrangement of a carbon skeleton by migration and degradation of the pyruvic acid side chain from *p*-hydroxyphenyl pyruvic (I) to homogentisic acid (VIII, Figure 12) is probably historically the first example of the rearrangement later designated as the 'NIH shift'. Its original formulation involving a cyclic peroxide IV[93] preceded by many years the proof of this mechanism which showed that both oxygen atoms from $^{18}O_2$ were incorporated into the substrate[94].

The *'NIH shift'* was an accidental discovery: the search for a rapid, clinically useful assay of phenylalanine hydroxylase, the enzyme deficient in phenylketonuric individuals, led to the synthesis of *p*-^{3}H-phenylalanine and to the realization that the enzymatically formed tyrosine still retained >90% of radioactivity. This observation, at first interpreted as an experimental error, was confirmed after the advent of NMR technique and instrumentation by repetition of the experiments with *p*-^{2}H-phenylalanine which gave *m*-^{2}H-tyrosine with 60% retention of deuterium[95].

What started by serendipity, was carried on by design: Arene oxides were shown to be substrates for the soluble enzymes of supernatant from liver microsomes[96]; the oxide of 4-^{2}H-toluene showed retention



of deuterium, i.e. the NIH shift, within the wide gamut known from model to microsomal hydroxylations[97]; finally, by the use of short incubation times and low temperatures (30 °C), the first isolation of an arene oxide, 1,2-naphthalene oxide, synthesized only weeks earlier[98] became possible[99]. By increasing the molar excess of glutathione in the presence of the conjugating enzyme, less *trans*-(1R,2R)-diol[100] and more glutathione conjugate was formed (Figure 13), proof that the arene oxide must be the precursor of both metabolites and that RSH is a better nucleophile than water. Hydration of the various arene oxides has only been observed as an enzymatic process. It is remarkable that water adds in the β-position, while the sulfur function ends up in the α-position of the naphthalene nucleus.

The spontaneous or acid-catalyzed rearrangement of the oxide can be carried out by photolysis to give

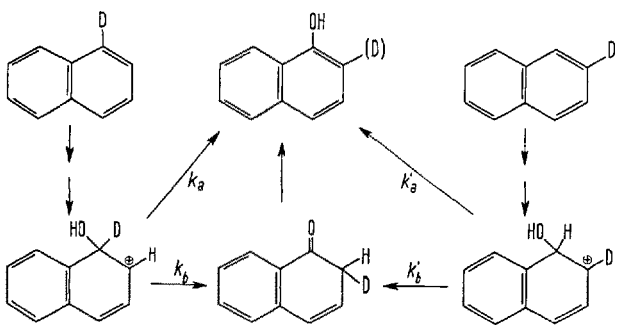


Fig. 14. $k_b \gg k_a$ and $k_b' \gg k_a'$.

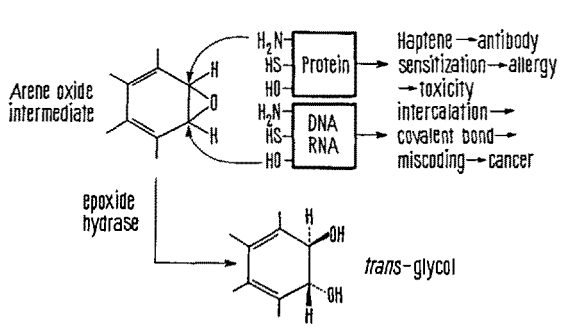


Fig. 15. The arene oxide as a potentially dangerous labile metabolite.

[88] B. WITKOP, Ann. Chem. *556*, 103 (1944).
[89] A. EK and B. WITKOP, J. Am. chem. Soc. *75*, 500 (1953); *76*, 5579 (1954).
[90] S. UDENFRIEND, C. T. CLARK and E. TITUS, J. Am. chem. Soc. *75*, 501 (1953).
[91] J. W. DALY and B. WITKOP, Angew. Chem. Int. Edn. *2*, 421 (1963).
[92] G. GUROFF, J. W. DALY, D. M. JERINA, J. RENSON, B. WITKOP and S. UDENFRIEND, Science *157*, 1524 (1967).
[93] S. GOODWIN and B. WITKOP, J. Am. chem. Soc. *79*, 179 (1957).
[94] B. LINDBLAD, G. LINDSTEDT and S. LINDSTEDT, J. Am. chem. Soc. *92*, 7446 (1970).
[95] G. GUROFF, C. A. REIFSNYDER and J. W. DALY, Biochem. Biophys. Res. Commun. *24*, 720 (1966).
[96] D. M. JERINA, J. W. DALY, B. WITKOP, P. ZALTZMAN-NIRENBERG and S. UDENFRIEND, Arch. Biochem. Biophys. *128*, 176 (1968).
[97] D. M. JERINA, J. W. DALY and B. WITKOP, J. Am. chem. Soc. *90*, 6523 (1968).
[98] E. VOGEL and F. G. KLÄRNER, Angew. Chem. Int. Edn. *7*, 374 (1968).
[99] D. M. JERINA, J. W. DALY, B. WITKOP, P. ZALTZMAN-NIRENBERG and S. UDENFRIEND, J. Am. chem. Soc. *90*, 6525 (1968); Biochemistry *9*, 147 (1970).
[100] D. M. JERINA H. ZIFFER and J. W. DALY, J. Am. chem. Soc. *92*, 1056 (1970).

α-dialone which is stable at the temperature of liquid nitrogen and rearranges to α-naphthol on warming to −80 °C[101].

More evidence on the keto tautomer as the precursor of α-naphthol comes from studies on α-deutero- and β-deutero-naphthalene (oxides): In either case microsomal hydroxylation leads to 2-deutero-1-naphthol with identical retention, i.e. ~70% (Figure 14)[102]. This keto tautomer must also be involved in the conversion of 1-^{3}H-naphthalene to 2-^{3}H-naphtho-1,4-quinone by oxotransition metal oxidants[102a]. The first direct conversion of an aromatic substrate to the arene oxide has been observed in the presence of pyridine oxides during photolysis[102b].

The existence of an intermediate as reactive as an arene oxide poses a certain danger in metabolism, where normally the epoxide hydrase system, recently solubilized and purified in this Laboratory[103] or the glutathione conjugases (glutathione S-epoxidetransferase)[104] act as built-in protectors. However, opening of such arene oxides by nucleophilic groups of biopolymers should lead to covalent linkages between aromatic nuclei and proteins or nucleic acids (Figure 15). The consequences could be very grave. Experiments with halogenated benzenes in rats have shown serious liver damage after stimulation of the liver microsomal system[105]. This aspect renews the need for long-range toxicity tests of known and new aromatic drugs. This picture is rounded off by the most recent findings that the chemically[106] or metabolically[107] accessible *K-region arene oxides* of benz(a)anthracene, dibenz(a,h)-anthracene and 3-methylcholanthrene were significantly more active in producing malignant transformations in several cell culture systems than the parent hydrocarbons[108]. The oxides are obtainable by heat-inactivation of the thermolabile epoxide hydrase by the same method that led to the isolation of squalene-2,3-oxide[109]. Their activity is probably the result of affinity-direction: these hydrocarbons may intercalate with the strands of nucleotides and then be kept in a position favorable for quasi-intramolecular opening of the epoxide by proximal nucleophilic groups. Similar affinity of proteins, such as the albumins, is known for many aromatic substrates.

[101] D. M. Jerina, O. Chapman, C. McIntosh and B. Witkop, J. Am. chem. Soc., in preparation.
[102] D. Boyd, D. M. Jerina and J. Daly, in preparation; cf. D. M. Jerina and J. W. Daly, *Mechanisms for the Oxidative Metabolism of the Aromatic Nucleus*, 2nd Int. Symposium on Oxidases and Related Oxidation-Reduction Systems, San Francisco 1970; Am. chem. Soc. Monographs, in press.
[102a] K. B. Sharpless and Th. C. Flood, J. Am. chem. Soc. *93*, 2316 (1971).
[102b] D. M. Jerina, D. R. Boyd and J. W. Daly, Tetrahedron Letters *6*, 451 (1970).
[103] F. Oesch and J. Daly, Biochim. biophys. Acta *227*, 692 (1971).
[104] B. Boyland and K. Williams, Biochem. J. *94*, 190 (1965).
[105] B. B. Brodie, W. D. Reid, A. K. Cho, G. Sipes, G. Krishna and J. R. Gillette, Proc. natn. Acad. Sci. *68*, 160 (1971).
[106] P. Sims, Biochem. J. *98*, 215 (1966). – M. S. Newman and S. Blum, J. Am. chem. Soc. *86*, 5598 (1964).
[107] J. K. Selkirk, E. Huberman and C. Heidelberger, Biochem. Biophys. Res. Commun., *43*, 1010 (1971).
[108] P. L. Grover, P. Sims, E. Huberman, H. Marquardt, T. Kuroki and C. Heidelberger, Proc. natn. Acad. Sci., USA, *68*, 1098 (1971).
[109] S. Yamamoto and K. Bloch, J. biol. Chem. *245*, 1670 (1970).

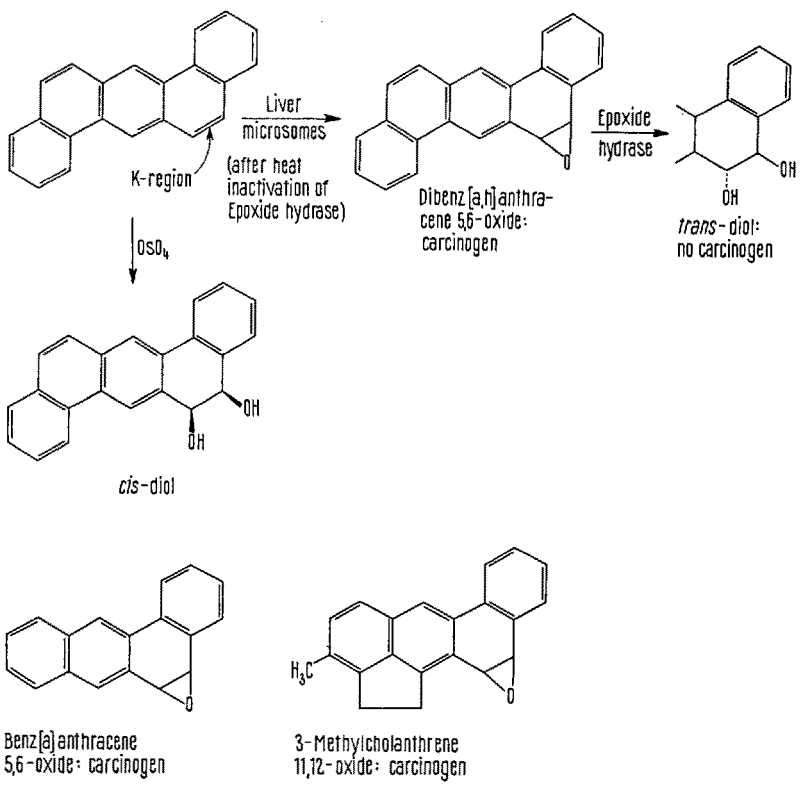


Fig. 16. All oxides of the K-region of the 3 cancerogenic hydrocarbons are 5–10 times more active in several cell culture systems in producing transformation to neoplastic tissue than the parent hydrocarbons. The *cis*- and *trans*-glycols at comparable concentrations are inactive.

I. *Epilogue*

Lack of time and space put an end to this exploratory excursion into the border areas of organic chemistry. Many a promising trail has been uncovered. Often the temptation was great to follow these new paths and run the risk of getting overcommitted to applications. Once a generally useful principle or method is found by the chemical pathfinder, follow-up and application should be up to the experts in the neighboring disciplines. By living like the artist on several planes simultaneously, the scientist should avoid the ominous trend toward overspecialization.

My modest role as a chronicler and narrator ends here, but my indebtedness to my younger collaborators and associates continues.

Zusammenfassung. Im Rückblick und Ausblick wird anhand einiger ausgewählter Arbeiten aus dem Laboratorium des Verfassers gezeigt, wie die Ziele und Methodologie der modernen Naturstoff-Forschung sich im Laufe der letzten 20 Jahre geändert haben. Noch immer kommen die unerwartetsten Anregungen für ungewöhnliche Strukturen aus der Natur, so zum Beispiel die neuen Tier-Alkaloide *Histrionicotoxin* und seine Begleiter, die an einem Spiro-cyclohexyl-piperidin-Skelett, Azetylen- und Allen-Seitenketten enthalten. Mehr noch als die Struktur interessiert beim *Batrachotoxin* die selektive Wirkung auf den passiven Transport von Natrium-Ionen durch Membranen elektrogener Gewebe, ein Effekt, der durch *Tetrodotoxin* reversibel aufgehoben werden kann. Heute zählt man auch die Eiweiss-Stoffe zu den wichtigen Naturprodukten. Ihre Erforschung wurde durch die Einführung selektiver Spaltungsmethoden ermöglicht und erleichtert, was am Beispiel des *Immunoglobulins* und des *Kollagens* gezeigt wird. Diese Spaltungsmethoden beruhen auf der Ausnutzung benachbarter Gruppen-Effekte, die weiterhin auch als Basis zum Ausbau von Enzym-Modellen dienen. Die Beschäftigung mit der Chemie der Überträgersubstanzen der Nervenfortleitung, wie Dopamin und Noradrenalin, führte zur Auffindung von *6-Hydroxydopamin*, das zum ersten Mal selektiv «chemische Sympathektomie» erlaubt. In der physiologischen Inaktivierung dieser Nervenhormone spielt die *Brenzkatechin O-Methyltransferase* eine grosse Rolle im Stoffwechsel, der man durch Reindarstellung und Beschreibung des Enzyms näherzukommen versucht. Im Stoffwechsel von aromatischen Substraten bedeutet die erste *Isolierung eines Arenoxyds* einen wichtigen Fortschritt. Ein derart labiles Stoffwechselprodukt spielt in der langfristigen Toxikologie aromatischer Arzneimittel und in der Ätiologie von Krebs durch cancerogene Kohlenwasserstoffe eine Rolle. Zum Studium von Oxydationsmechanismen mikrosomaler oder Modell-Hydroxylierungen wird als Kriterium mehr und mehr die sogenannte *NIH-Verschiebung* herangezogen.

SPECIALIA

Les auteurs sont seuls responsables des opinions exprimées dans ces brèves communications. – Für die Kurzmitteilungen ist ausschliesslich der Autor verantwortlich. – Per le brevi comunicazioni è responsabile solo l'autore. – The editors do not hold themselves responsible for the opinions expressed in the authors' brief reports. – Ответственность за короткие сообщения несёт исключительно автор. – El responsable de los informes reducidos, está el autor.

Synthesis of a Structure Proposed for Scotophobin

Scotophobin[1], a behavioral 'memory code word', has been formulated as the pentadecapeptide[2] Ac-ser-asp-asn-asn-glu-gln-gly-lys-ser-ala-glu-gln-gly-gly-tyr-NH_2. A synthesis of this compound is now reported[3,4], as well as full biological data.

N-Benzyloxycarbonyl-O-*t*-butyl-tyrosine (I) was converted into a mixed anhydride (N-methyl morpholine and isobutyl chloroformate) and treatment with ammonia produced the amide (II). In turn, hydrogenation formed the amine (III); a coupling to N-benzyloxycarbonyl-glycine (IV) by the mixed anhydride procedure gave the dipeptide (V). Hydrogenation then afforded the S_{14-15} amine glycyl-O-*t*-butyl-tyrosinamide (VI).

The sequence S_{10-13} was prepared beginning with methyl glycinate (VII) and N-benzyloxycarbonyl-glutamine (VIII), which on joining by the mixed anhydride procedure, yielded methyl N-benzyloxycarbonyl-glutaminyl-glycinate (IX). Hydrogenation furnished the amine (X); a combination with N-benzyloxycarbonyl-γ-*t*-butyl- glutamic acid (XI) by the mixed anhydride method produced the tripeptide (XII). Hydrogenation formed the amine

[1] G. UNGAR, *Molecular Mechanisms in Memory and Learning* (Plenum Press, New York 1970), p. 149.

[2] G. UNGAR, personal communication, February 24, 1970

[3] A stepwise preparation of the scotophobin sequence was discussed in detail by B. WEINSTEIN at The Second American Peptide Symposium, Cleveland, Ohio, August 1970; the biological results for the tridecapeptide S_{3-15} were mentioned, too. Additional information on this synthesis was presented at the 160th National Meeting of the American Chemical Society, Chicago, Illinois, September 1970, Abstracts ORGN 008.

[4] The original route met with two major hurdles: The blocked peptides beginning with the subunit S_5-S_{15} are very insoluble and thus slow to react, while some fragments containing the S_{3-4} asn-asn sequence undergo facile hydrolytic cleavage.