Biochemical Pharmacology, Vol. 16, pp. 537–551. Pergamon Press Ltd. 1967. Printed in Great Britain

BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—IX

UNCOUPLING OF OXIDATIVE PHOSPORYLATION AND INHIBITION OF A THIOL ENZYME (PAPAIN) BY SOME CYCLIC β-DIONES AND NINHYDRIN

M. W. WHITEHOUSE* and JEAN E. LEADER

Department of Biochemistry, University of Oxford†

(*Received* 16 *July* 1966; *accepted* 20 *October* 1966)

Abstract—Some novel 2-acyl-indan-1,3-diones were synthesized and characterized. These and other acyl-indandiones were tested for uncoupling activity using rat liver mitochondria (oxidizing succinate). *o*.Hydroxybenzoyl-indandiones, which readily cyclized to yield chromones during their preparation, also uncoupled oxidative phosphorylation.

The uncoupling potency of acyl-indandiones, pyrazolidinediones and certain acidic pyrazolones generally paralleled (a) their lipophilic character and (b) their avidity for protein ε-lysyl amino groups (as indicated by the compound's ability to inhibit the 2,4,6-trinitrobenzaldehyde–albumin reaction). *p.p'*-Dichlorophenylbutazone, the 4-butyryl analogue of phenylbutazone and the following indandiones = 2-isoheptanoyl-2-*p*.chlorphenyl-, 2-(3′,4′)dichlorobenzoyl-, were the most active uncoupling agents found in this limited survey. The anomalous (low) uncoupling activity and lysyl-binding activity of certain *ortho* substituted benzoyl-indandiones could be attributed to steric hindrance.

Some limitations of the trinitrobenzaldehyde-albumin reaction for screening compounds for potential drug activity are disclosed, notably (i) reaction of the aldehyde with compounds containing an active methylene group (e.g. unsubstituted β-diones like indan-1,3-dione itself), and (ii) where the drug under investigation may bind to plasma albumin at sites other thsn lysine amino groups (e.g. sulphinpyrazone). Sulphamethazine potentiates the reaction between trinitrobenzaldehyde and bovine plasma albumin.

Ninhydrin, hydrindantin and some related non-acidic aromatic 1,2 and 1,4-diones also uncoupled oxidative phosphorylation, but only in the absence of added thiols, and did not inhibit the trinitrobenzaldehyde–albumin reaction (in those instances where these diones did not react with either the albumin or the trinitrobenzaldehyde). These compounds, and also phenylbutazone and some of its analogues, were potent inhibitors of the esterase activity of crystalline papain in the presence of excess thiol.

It is postulated that non-acidic diones may uncouple oxidative phosphorylation by reacting with essential thiol groups involved in mitochondrial energy conservation, whereas acidic uncoupling drugs primarily inhibit mitochondrial phosphorylation by "neutralizing" essential amino groups.

PHENYLBUTAZONE (1,2-diphenyl-4-*n*.butyl-pyrazolidine-3,5-diones, Butazolidine®) and 2-phenyl-indan-1,3-dione (Phenindione) are anti-inflammatory drugs[1,2] able to uncouple oxidative phosphorylation[3–5]—a property also exhibited by other acidic

* Present address: Department of Experimental Pathology, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T. Australia.

† Address for reprints.

anti-inflammatory/antirheumatic drugs.[6] This report describes some studies of the relationship between uncoupling activity *in vitro*, chemical structure and certain physiochemical properties, in a series of phenylbutazone and indandione derivatives and analogues.

EXPERIMENTAL

A. *Chemical*

Diethyl mesoxalate was prepared from diethyl malonate by oxidation with nitrous fumes.[7] Pyrazolones and other diones were obtained from commercial supply houses and from Dr. R. Pfister (J. R. Geigy, A.G., Basle), Dr. K. Trnavsky (Piestany, Czechoslovakia), Mr. D. J. Drain (Smith & Nephew Research, Harlow), Dr. P. B. Fowler (Geigy Pharmaceuticals, Manchester) and Dr. E. Jucker (Sandoz, A.G. Basle). Diphenadione (U. 1363) was donated by the Upjohn Co. (G.B.). The preparation and characterization of some other indan-1,3-dione derivatives is given below.

2-Isobutyryl- and 2-isovaleryl-indandiones were synthesized from diethyl phthalate and the appropriate methyl ketones.[8] Extension of this method using 6-methylheptan-2-one (b.p. 161–162°) synthesized from ethyl acetoacetate and isamylbromide, gave 2-isoheptanoyl-indan-1,3-dione m.p. 30–31° recrystallized from aqueous methanol ($C_{16}H_{18}O_3$ requires C, 74·5%; H, 6·95%; found C, 74·0%; H, 7·0%).

2-(4′-*Hydroxybenzoyl*)-*indandione* was prepared from 4-hydroxyacetophenone using excess diethyl phthalate as the solvent,[9] with m.p. 280–282° (dec.).

2-(2′,4′-*Dimethoxybenzoyl*)-*indan*-1,3-*dione* was prepared similarly from 2,4-dimethoxyacetophenone. The product was recrystallized from aqueous ethanol, m.p. 168–169°. ($C_{18}H_{14}O_5$ requires C, 69·7%; H, 4·55%; found C, 69·7%; H, 4·50%).

2-(2′,6′-*Dimethoxybenzoyl*)-*indan*-1,3-*dione* was prepared similarly from 2,6-dimethoxyacetophenone (m.p. 68–69°, obtained by methylating 2,6-dihydroxy-acetophenone[10]). The product was recrystallized from chloroform, m.p. 240° (dec.). ($C_{18}H_{14}O_5$ requires C, 69·7%; H, 4·55%; found C, 69·9%; H, 4·78%).

2,3-(2′,3′-*Indene*-1′-*one*)-*chromone* was obtained when diethyl phthalate was reacted with 2-hydroxy-acetophenone, being formed by internal cyclization of the expected product, 2-salicyl-indan-1,3-dione when the reaction mixture was acidified to precipitate the product. The yellow chromone was recrystallized from ethanol, m.p. 238° ($C_{16}H_8O_3$ requires C, 77·4%; H, 3·23%; found C, 77·4%; H, 3·39%). It gave no colour with alcoholic ferric chloride but if an alkaline solution of this chromone was acidified and immediately extracted with ether, the ethereal extract gave the intense reddish-brown coloration characteristic of 2-acyl-indan-1,3-diones. Solutions of the chromone in alkali (pH 10) after standing for a few min absorbed light at 285 and 330 mμ (characteristic of 2-acyl-indan diones).

2,3-(2′,3′-*Indene*-1′-*one*)-7-*hydroxychromone* was prepared by demethylation of 2-(2′,4′,dimethoxybenzoyl)-indan-1,3-dione with 5 equiv. of anhydrous aluminium chloride in boiling benzene. After 2 hr the mixture was cooled, poured over ice, stirred and filtered when the ice had melted. The yellow product was recrystallized from 2-ethoxyethanol. It began to decompose at 300° but had not melted at 360°. The existence of polymeric intermolecular H bands in the crystalline state was indicated by a broad band at 3·10 μ in the i.r. spectrum (Nujol mill) of the solid product ($C_{16}H_8O_4$ requires C, 72·9%; H, 3·04%: found C, 72·4%; H, 3·49%). This product gave an intense coloration with alcoholic ferric chloride under the same conditions

as the 7-desoxychromone (above) and there was a similar change in the u.v. spectrum at pH 10·0 due to ring opening → 2-acylindan-1,3-dione.

2,3-(2′,3′-*Indene*-1′-*one*)-5-*hydroxychromone* was prepared similarly by demethylating 2-(2′,6′-dimethoxybenzoyl)-indan-1,3-dione. The yellow product was recrystallized from 2-ethoxyethanol, m.p. 298–300° (dec.). ($C_{16}H_8O_4$ requires C, 72·9%; H, 3·04%: found C, 71·0%, H, 3·45%). It gave a reddish brown coloration with alcoholic ferric chloride under the same conditions as the above two compounds and showed similar changes in u.v. absorption at pH 10·0.

2-(2′,4′-*Dimethylbenzoyl*)-*indan*-1,3-*dione* prepared from 2,4-dimethyl-acetophenone under the standard conditions[9] was a yellow solid when recrystallized from ethanol, m.p. 100–102° ($C_{18}H_{14}O_3$ requires C, 77·7%; H, 5·07%: found C, 77·7%; H, 5·28%).

2-(3′,4′-*Dichlorobenzoyl*)-*indan*-1,3-*dione* prepared from 3,4-dichloro-acetophenone was a pale yellow solid, m.p. 192–194° (from ethanol). ($C_{16}H_8Cl_2O_2$ requires C, 60·2%; H, 2·53%; Cl, 22·2%: found C, 60·8%; H, 2·76%; Cl, 22·1%).

2-(2′,4′,6′-*Trimethylbenzoyl*)-*indan*-1,3-*dione* prepared from 2,4,6-trimethylacetophenone was a red oil, sp. gr. 1·55, decomposing above 200°. ($C_{19}H_{16}O_3$ requires H, 5·48%; found 5·34%). It gave the characteristic red coloration with ferric chloride for an indandione.

Bis-indan-1,3-*dione* was prepared by heating ninhydrin with sulphuric acid, m.p. 295°.

Indan-1,2,3-*trione*-2-*oxime* was prepared by the action of nitrous acid on indan-1,3-dione,[12] m.p. 204° (from glacial acetic acid).

4-*Acetyl*-1-*phenyl*-3-*methyl-pyrazol*-5-*one* was prepared by acetylation of 1-phenyl-3-methyl-pyrazolone with acetic anhydride,[13] m.p. 59° (from aqueous ethanol). It gave a ruby red coloration with ethanolic ferric chloride.

B. *Biochemical*

These and other compounds were added as solutions in 25 μl or 50 μl N.N.-dimethylformamide (DMF)* to rat liver mitochondria respiring on succinate (30 mM). The P/O ratio in the presence and absence of the drugs was determined exactly as described.[5] Values for P/O ratio given in the tables were mean of two determinations (agreeing within 10 per cent). Active uncoupling compounds were also tested for their effect on yeast hexokinase[14] (used to trap newly-synthesized ATP).

Drug association with protein (lysyl) amino groups was determined colorimetrically using 2,4,6-trinitrobenzaldehyde and bovine plasma albumin[15,16] as follows. The drug was dissolved in DMF and diluted (usually ten-fold) with 0·1 M phosphate buffer, pH 7·4 to give a 3 mM drug solution. One ml of this solution was mixed with 1 ml 2% (w/v) serum albumin (Armour Pharmaceutical Co., Eastbourne) in the same buffer and 1 ml of 0·1 mM trinitrobenzaldehyde (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.): 7·2 mg aldehyde recrystallized from benzene, dissolved in 1 ml DMF and diluted to 100 ml with 0·1 M phosphate buffer). After 30 min at room temperature the optical density of the aldehyde–albumin complex was measured at 425 mμ and 525 mμ. Controls with drug but no albumin and controls with drug but no aldehyde were run in addition to drug-free controls.

* Abbreviations used: DMF—dimethylformamide; EDTA—diaminoethane-tetracetic acid; P_i—inorganic phosphate; ATP—adenosine-5′triphosphate; BAEE—N-benzoyl-L-Arginine ethyl ester; TNBal—2,4,6-Trinitrobenzaldehyde.

The enzyme activity of crystalline papain (Sigma Chemical Co., London) was measured at room temperature with a pH-stat (Radiometer, Copenhagen) by the addition of 10 mM sodium hydroxide in 0·15 M sodium chloride, using N-benzoyl-L-arginine ethyl ester as substrate (2 mM) at pH 6·5 in a mixture of 10 mM sodium citrate, 150 mM sodium chloride and 1% (v/v) DMF. Papain (1·6 mg/ml) was preincubated at room temperature with 20 mM disodium EDTA, 25 mM mercaptoethanol and 20 mM sodium citrate (pH 6·5) for at least 15 min. Fifty microlitres of this activated enzyme solution was added to 5 ml incubation mixture containing the drug (added in 50 μl DMF), incubated for at least 3 min (and the pH readjusted to 6·5) before adding the substrate (0·1 ml 0·1 M BAEE). The liberation of protons over the next 5 min was recorded and then a further 50 μl of enzyme was added and the rate of proton liberation in each successive minute was also recorded.

RESULTS AND DISCUSSION

(Compounds listed in the Tables did not inhibit mitochondrial respiration at the concentrations given there, unless otherwise noted).

Uncoupling by indan-1,3-diones (Table 1)

p. Chlorophenyl-indandione (Indalitan®), an anticoagulant drug, was more potent than phenindione in uncoupling oxidative phosporylation and considerably more lipophilic, as indicated in Table 2 by its higher partition coefficients. These coefficients obtained for a range of water-immiscible solvents chosen arbitrarily to differ in polarity, indicate the relative ability of individual compounds (in a series of related compounds) to pass from a neutral aqueous solution into an organic phase. It is presumed that these coefficients might also indicate the relative ability of these compounds to pass into the lipid-rich phase of mitochondria where phosphorylation and energy-conservation (linked to electron transport) takes place.

In a series of indandiones substituted at C-2 with alkanoyl groups, the individual members had similar dissociation constants (p*K*'s) but varied widely in lipophilic character, as indicated by their partition coefficients (Table 2). Their uncoupling potency paralleled this lipophilic character. 2-Isoheptanoyl-indandione, the most lipophilic compound of those examined in this series, was also the most active in uncoupling oxidative phosphorylation being comparable with *p.* chlorophenyl-indandione and 2,4-dinitrophenol in potency (Table 1) *in vitro*. Diphenylacetyl indandione (diphenadione), another commercial anticoagulant drug, was less potent than these two indandiones in uncoupling oxidative phosporylation.

In a related series of 2-benzoyl-indandiones, the presence of chlorine or not more than 2 methyl groups on the phenyl nucleus potentiated uncoupling activity and increased the partition coefficients (Table 2). Conversely, substituting a 4-hydroxyl group or 2,4- and 6-methoxy groups in the phenyl nucleus diminished both the uncoupling activity (Table 1) and lipophilic character as reflected by these partition coefficients, (Table 2). All these substituents increased the pK_a. Evidently, lipophilic character, rather than acidity, primarily determines the uncoupling potency of these particular 2-acyl-indandiones where the acyl group is either aliphatic or aromatic in character.

Attempts to prepare 2-*o*.hydroxybenzoyl derivatives of indandione were hindered by the readiness with which they cyclized to form chromones. This cyclization is

TABLE 1. UNCOUPLING OF OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA BY 2-SUBSTITUTED INDAN-1,3-DIONES

Substituent (at C-2)	Conc. (mM)	P/O (%)	Substituent	Conc. (mM)	P/O (%)
Phenyl	0·1	25	Diphenylacetyl	0·1	0
				0·05	65
p.Chorphenyl	0·025	25			
	0·01	70	Benzoyl	0·5	30
				0·25	75
None*	0·5	80			
	1·0	0	4-Hydroxybenzoyl	0·5	75
Nitro	2·5	88	2-Hydroxybenzoyl†‡	0·5	10
Acetyl‡	0·25	90	2,4-Dihydroxybenzoyl†	1·0	100
	0·5	50			
			2,6-Dihydroxybenzoyl†	1·0	60
Isobutyryl	0·25	60			
			2,4-Dimethoxybenzoyl‡	1·0	25
Isovaleryl‡	0·05	60			
			2,6-Dimethoxybenzoyl‡	5·0	100
Isoheptanoyl‡	0·05	5			
			2,4-Dimethylbenzoyl	0·2	50
	0·025	20			
	0·01	70	3,4-Dimethylbenzoyl	0·2	45
(2,4-Dinitrophenol	0·025	40	2,4,6-Trimethylbenzoyl	0·2	100
	0·01	65)		1·0	50
			3,4-Dichlorobenzoyl	0·05	15

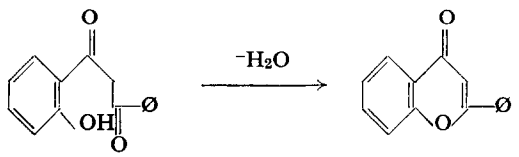
Phosphorylation quotient (P/O ratio) given as % that in drug-free controls.
* i.e. Indan-1,3-dione itself.
† Added as the corresponding 2,3-(2′,3′-indene-1′-one) chromone—see Experimental.
‡ Also partially inhibited respiration (>20%).

TABLE 2. PHYSICOCHEMICAL PROPERTIES OF SOME 2-SUBSTITUTED INDAN-1,3-DIONES

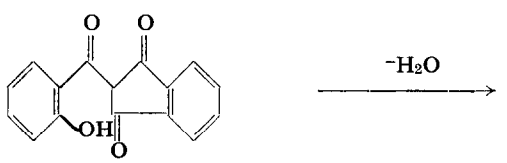
Substitutient (at C-2)	p*Ka** ± 0·1	Partition coefficient		
		n octanol	Tri-*n* butyl phosphate	Chloroform
None†	7·2 (a)	1·4		
Nitro	1·7			
Phenyl	4·4 (a)	2·0		
p. Chlorphenyl	4·0 (a)	13	31	8·5
Acetyl	2·9 (a)	0·3	1·6	0·4
Isobutyryl	3·2 (a)	1·9		
Isovaleryl	3·3 (a)	4·3	20	28
Isoheptanoyl	3·5 (a)	21		
Benzoyl	2·7 (b)	0·6	6·3	3·4
4′-Hydroxybenzoyl	3·4 (b)	0·45		
2′,4′-Dimethoxybenzoyl	3·9 (c)	0·53	3·0	13
2′,6′-Dimethoxybenzoyl	3·5 (c)	0·26	0·6	1·7
2′,4′-Dimethylbenzoyl	3·8 (a)	4·0	12	8·5
3′,4′-Dimethylbenzoyl		7·7	9·2	5·5
2′,4′,6′-Trimethylbenzoyl	4·6 (c)	9·6	15	8
3′,4′-Dichlorobenzoyl		15	25	3·7
2′-Hydroxybenzoyl‡		1·2		
2′,4′-Dihydroxybenzoyl‡		2		
2′,6′-Dihydroxybenzoyl‡		>2		
(2,4-Dinitrophenol	4·0 (a)	0·05	2·3	0)

Acid dissociation constants (p*Ka*'s) measured in aqueous ethanol. Partition coefficients are for distribution of drug between neutral salt solution (pH 7) and organic solvent specified.
* Letters in brackets refer to ethanol concentration = 1% (a), 33% (b) or 50% (c).
† i.e. Indan-1,3-dione itself.
‡ Added as chromones.

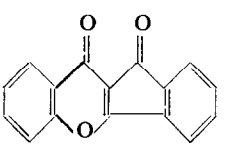
analagous to that utilized in the preparation of flavone from 2-hydroxy-dibenzoylmethane (Fig. 1). Solution of these chromones at pH 7 were in equilibrium with the corresponding indandione (indicated by the u.v. absorption and ferric chloride colour reaction) and uncoupled oxidative phosphorylation. Solutions containing salicylindandione (and the chromone) were more potent than both benzoyl- and 4-hydroxybenzoyl-indandione in uncoupling oxidative phosphorylation, indicating successful fusion of the two uncoupling pharmacophores namely, the indandione and salicyl structures.



2-hydroxy dibenzoyl methane flavone



2-(2′-hydroxybenzoyl)-indan-1,3-dione



2,3-(2′,3′-indene-1′-one)chromone

FIG. 1. Cyclization of 2-hydroxy dibenzoyl methane and 2-(2′-hydroxybenzoyl)-indan-1,3-dione.

Attempts to improve upon this modest achievement, by combining the β-resorcyl and γ-resorcyl uncoupling pharmacophores[17] with indandione, were not successful—the extra hydroxyl group diminished the lipophilic character and in the case of 2,6-dihydroxybenzoyl-indandione, may have sterically blocked interaction between the active enol group at C-1 (or C-3) and the mitochondrial drug-receptor.

Evidence for such steric hindrance was also obtained with 2,6-dimethoxybenzoyl and 2,4,6-trimethylbenzoyl-indandiones, which were rather less potent in uncoupling oxidative phosphorylation than their lipophilic character would suggest (Table 2). Furthermore these two compounds and 2,6-dihydroxybenzoyl-indandione were each bound less strongly by the amino groups of lysine residues in plasma albumin, than either benzoyl- or salicyl-indandiones (Table 3).

Collectively these findings indicate that uncoupling activity of indandiones is largely determined by the availability of the enol group for anionic binding and the lipophilic character of the whole molecule.

Uncoupling by ninhydrin (indan-1,2,3-trione hydrate) and related compounds (Table 4)

Ninhydrin was noted to be a rather potent uncoupling agent but a very weak acid (p*K*, 9·0).[5] It is toxic to animals,[18,19] as might be expected of a rather potent uncoupling agent (but also see below).

This uncoupling action of ninhydrin was almost completely reversed on diluting the drug, in experiments carried out as follows. Lightly buffered mitochondria (in

TABLE 3. DRUG-BINDING BY PROTEIN LYSYL GROUPS AS INDICATED BY COMPETITION WITH TRINITROBENZALDEHYDE FOR THESE GROUPS

Compound	Conc. (mM)	% Inhib.	2-Substituted indan-1,3-dione	Conc, (mM)	% Inhib.
None	—	0			
Alloxan	1·0	6	2-Phenyl-	0·5	20
1,3-Diphenyl-2,4,5-trioxocyclopentane	1·0	68	2-Nitro-	1·0	35
Menadione	1·0	12	2-Acetyl-	0·5	40
1,2-Naphthoquinone	1·0	<20	2-Isoheptanoyl	0·5	58
Dehydroacetic acid	1·0	20	2-Diphenylacetyl-	0·5	62
Sulphamethazine	1·0	−45†	2-Benzoyl	0·5	53
Sulphathiazole	1·0	22	2-Salicyl*	0·5	35
Chloramine-T	1·0	0	2-(2′,4′-Dimethoxybenzoyl)	1·0	37
			2-(2′,6′-Dimethoxybenzoyl)	1·0	6
			2-(3′,4′-Dichlorobenzoyl)	0·5	55
			2-(2′,4′,6′-Trimethylbenzoyl)	0·5	28

0·1 mM Plasma albumin, 0·1 ml trinitrobenzaldehyde (TNBal) and drug mixed at pH 7·4 and light absorption at 425 mμ and 525 mμ, due to albumin–TNBal complex, determined after 30 min at room temperature. Quenching (usually measured at 425 mμ) by drug = % inhibition.

* Solution of the corresponding chromone in phosphate buffer pH 7·4.

† i.e. Stimulation of trinitrobenzaldehyde–albumin interaction.

10 mM phosphate) were pre-incubated with ninhydrin in the side arm of a Warburg flask (total fluid volume = 0·25 ml) for 10 min at 30°, then diluted ten-fold by tipping into the main compartment of the flask which contained the normal incubation medium and hexokinase. Phosphorylation quotients of 1·3–1·5 were obtained with succinate as substrate when the initial ninhydrin concentration was 0·2 mM, subsequently diluted to 0·02 mM: parallel incubations with no ninhydrin and 0·2 mM ninhydrin (final concentration) gave phosphorylation quotients of 1·6–1·7 and 0·0 respectively. Higher levels of ninhydrin also inhibited mitochondrial respiration (40–80 per cent inhibition with 2·5 mM ninhydrin) but this effect was also completely reversed by dilution. Mitochondria pre-incubated with 4 mM ninhydrin (and then subsequently diluted ten-fold) took up oxygen at the same rate as parallel incubations of mitochondria without ninhydrin.

Addition of borate ions (2·5 mM) to aqueous solutions of ninhydrin (50–500 μM) liberates protons, due to complex formation[20] but had little effect on the uncoupling potency (only diminishing the uncoupling effect of 50 μM ninhydrin). Boiling ninhydrin (0·5 mM) with excess glycine to form the characteristic purple coloration, Ruhemann's purple,[18] abolished the uncoupling activity. The uncoupling activity of 0·2 mM ninhydrin was almost completely abolished by adding to the incubation medium any of the following compounds: 1 mM *o*.phenylene-diamine or cysteamine, 2·5 mM cysteine ethyl ester or D.L. penicillamine, 5 mM cysteine or mercaptoethanol

or glutathione or thioglycollate salts. A number of other amino compounds did not prevent ninhydrin from uncoupling phosphorylation including 5 mM L-proline, imidazole, alanine, arginine methyl ester, lysine methyl ester. Cysteamine, thioglycollates and *o*. phenylene-diamine (at 5 mM) did not reverse the uncoupling action of acidic diones (1 mM indan-1,3-dione, 0·5 mM phenylbutazone, 0·2 mM 2-phenylindan-1,3-dione) or 2,4,6-trinitrobenzaldehyde (0·5 mM).

TABLE 4. EFFECT OF NINHYDRIN AND SOME RELATED DIONES UPON OXIDATIVE PHOSPHORYLATION

Compound	Conc. (mM)	P/O (%)	Compound	Conc. (mM)	P/O (%)
None	—	100			
Ninhydrin	0·2	0	Hydrindantin	0·2	10
	0·1	45		0·1	30
	0·05	60		0·05	60
Indan-1,2,3-trione-2-oxime	0·5	55	Bisindan-1,3-dione	0·5	90
"Ruhemann purple"	0·5	100	Anhydro-bis-indan-1,3-dione	0·1	40
Isatin	1·0	100	1,2-Napthoquinone	0·8*†	0
Benzoylformic acid	2·5	100	1,2-Naphthoquinione-4-sulphonic acid	1·0	20
Phenylglyoxal	1·0*	100		0·5	85
1,3-Diphenyl-propan-1,2,3-trione	1·0*	95	Chloramine-T	1·0	50
Diethyl mesoxalate	5·0	100	1,3-Diphenyl-2,4,5-trioxocyclopentane	0·25	35†
Phthalaldehyde	0·2	40–75‡	Menadione	0·5*	65

P/O ratio in presence of drug given as percentage P/O ratio with drug-free conrols.
* Sat. solutions.
† Also inhibited respiration at this concentration.
‡ See text.

A number of 1,2-dioxo and 1,2,3-trioxo compounds were examined for ninhydrin-like activity (Table 4). Only rhodizonic acid and alloxan exhibited similar uncoupling activity but alloxan also inhibited the yeast hexokinase at those concentrations ($\geqslant$2 mM) at which it appeared to uncouple oxidative phosphorylation. However, alloxan did stimulate the mitochondrial ATPase activity[5] at pH 7·4 (tris-hydrochloride buffer) and this is confirmed by an independent report.[21] (Concentrations of ninhydrin less than 1 mM had no effect on the activity of the yeast hexokinase used in these experiments, and ninhydrin is reported to be less potent than alloxan as an inhibitor of muscle hexokinase[22]). *o*-Phthaldehyde consistently inhibited respiration (at concentrations $>$ 0·2 mM) but showed rather variable uncoupling activity with different preparations of mitochondria (due to oxidation?).

Isatin, chloramine-*T* and 1,2-naphthoquinone which react like ninhydrin with aminoacids and proteins[23,24] either did not uncouple phosphorylation or were less active than ninhydrin in this respect.

The reduction product (pinacol) of ninhydrin, hydrindantin, which dissociates (in alkali) to give ninhydrin and 2-hydroxy-indan-1,3-dione,[18] proved to be approximately equipotent with ninhydrin and its uncoupling activity was also abolished by coincubation with thioglycollate, cysteamine or *o*. phenylene-diamine. These findings suggest that the uncoupling activity of ninhydrin cannot be simply ascribed to its reduction

products, 2-hydroxy-indandione or hydrindantin (which are probably more acidic than ninhydrin itself) possibly formed by interaction between ninhydrin and mitochondrial thiols. (Hydrindantin should be twice as potent as ninhydrin if either it or 2-hydroxyindandione were the true uncoupling species and if ninhydrin was only effective following reduction *in situ*.)

Menadione (2-methyl-1,3-napthoquinone) exhibited some uncoupling activity and has been shown to inhibit the ATP:P_i exchange reaction in rat liver mitochondria.[25] This compound and 1,2-napthoquinone did not compete with trinitrobenzaldehyde for the lysyl ϵ-amino groups on plasma albumin, as did acyl-indandiones and other uncoupling β-diones (see previous and following sections)—suggesting that 1,2- and 1,4-diones might influence mitochondrial phosphorylation through some other mechanism than combination with a reactive amino group.

This trinitrobenzaldehyde–albumin reaction could not be used to study the interaction of albumin amino groups with ninhydrin, hydrindantin or 1,3-diphenyl-propan-1,2,3-trione as these compounds either reacted with trinitrobenzaldehyde (TNBal) alone or, in the presence of both TNBal and plasma albumin, reacted abnormally to give products which absorbed more light at 525 mμ than at 425 mμ. (The TNBal-albumin reaction without drugs, or with acidic anti-inflammatory drugs present, yields chromophores which absorb light more strongly at 425 mμ than at 525 mμ).[16]

Uncoupling by phenylbutazone and some pyrazolones

Table 5 gives data which indicate some factors contributing to the potency of phenylbutazone in uncoupling oxidative phosporylation and binding to the lysyl amino groups of plasma albumin. Lipophilic substituents in the phenyl nuclei (chlorine, trifluoromethyl) potentiate both these activities. Hydrophilic substituents (hydroxyl, carboxyl) diminish the uncoupling activity but not the albumin-binding. Increasing the acidity of the molecule by changing the substituent at C-4 diminishes both the uncoupling activity and albumin binding (e.g. sulphinpyrazone or "ketazon"), except where the acidgenic substituent is an α-carbonyl function (G. 28551) giving a "double β-dione" with both endocyclic and exocyclic β-dione groupings.

This parallels observations with resorcinols (in tautomeric equilibrium with β-diketodihydrobenzenes) and the indan-1,3-diones, acylation of which (at the methylene carbon between the dioxy groups yielding double β-diones = γ-resorcyl derivatives or acylindandiones), usually decreases the pK and potentiates both the uncoupling activity[17] and combination with protein (lysyl) amino groups (Whitehouse, unpublished observations). Dehydroacetic acid (pK 5·8 in 50% DMF), the simplest readily available example of such a double β-dione, probably has insufficient lipophilic character to exhibit useful uncoupling activity or bind strongly to albumin amino groups (see Table 3).

The feeble uncoupling activity of the desbutyl (G. 14744) and (mono) desphenyl (G. 29682) analogues of phenylbutazone, indicates the importance of these lipophilic groups for optimal combination of the drug with reactive sites of proteins.

The dione structure is evidently not an absolute requirement for uncoupling activity since certain lipophilic acidic pyrazolones also uncouple mitochondrial phosphorylation and bind to lysyl amino groups (Table 5). A dinitro derivative of 1-phenyl-3-methylpyrazol-5-one, picrolonic acid is too acidic (cf. 2-nitro-indandione

TABLE 5. (*i*) UNCOUPLING OF OXIDATIVE PHOSPHORYLATION AND (*ii*) BINDING TO PLASMA ALBUMIN, BY SOME PYRAZOLIDINE-3,5-DIONES (PDO) AND PYRAZOL-5-ONES (PO)

Compound	Code no. (alt. name)	Concn. (mM)	P/O (% control)	Alb. TNBal (% inhib)	pK_a*
None	—	—	100	0	
4-*n*. Butyl-1-Phenyl-PDO	G.29682	2·5	65	28	6·5
4-*n*. Butyl-1,2-diphenyl-PDP	Phenylbutazone	0·2	55	45	5·3
		0·5	0		4·5 (W)
(Mono)-O-Methyl-PB	—	0·2	92	—	—
4-*n*. Butyl-1,2-di(*p*. chlorphenyl)PDO	G.15140	0·05	63	72	4·7
4-*n*. Butyl-1,2-di(*p*. α′α′α′-trifluoro-tolyl)-PDO	FBU.85	0·1	65	75	3·5
		0·2	0		
1,2-Diphenyl-PDO	G.14744	1·0	40	†	5·4
4-Ethyl-1,2-diphenyl-PDO	G.15138	1·0	57	50	5·5
4-Isopropyl-1,2-diphenyl-PDO	G.13838	1·0	70	40	6·4
4-*n*. Hexyl-1,2-diphenyl-PDO	G.27509	0·1	37	48	5·5
4-Butyryl-1,2-diphenyl-PDO	G.28551	0·05	45	60	3·3
4-(3′oxobutyl)-1,2-diphenyl-PDO	"Ketazon"	1·0	55	16	4·3
1,4-Diphenyl-PDO	G.28027	2·5	55	10	4·4
4-(2′-Phenylsulphinylethyl)-1,2-diphenyl-PDO	Sulphinpyrazone	0·5	53	0	2·8 (W) 3·8
4-*n*. Butyl-1-phenyl-2-*p*. hydroxy-phenyl-PDO	Oxyphenbutazone	0·2	67	48	5·9
4-*n*. Butyl-1-phenyl-2-*p*. carboxyphenyl-PDO	HP 433	0·8	80	48	
4-*n*. Butyl-1,2-di(*p*. carboxyphenyl)-PDO	HP 358	5·0	80	46	
1-Phenyl-3-methyl-PO		5·0	68	†	7·9 (D)
1-Phenyl-3-methyl-4-acetyl-PO		2·0	35	22	4·0 (D)
Picrolonic acid		5·0	100	70	2·4 (W)
1-*p*. Nitrophenyl-3-methyl-PO		0·2	50	†	6·8
1,3-Diphenyl-PO		0·1	55	†	6·2 (W)

Albumin binding measured by displacement of 2,4,6-trinitrobenzaldehyde (TNBal) with 1 mM drug (cf. Table 3). Data on pK_a's of PDO's in G. series, determined in 80% aq. methylcellosolve (MC), kindly supplied by Dr. R. Pfister, Basle. Other enolic pK_a's taken from literature[31,32] or determined by direct titration.

* In 80% MC except where stated; W = water, D = 50% aq. N,N-dimethylformamide.

† Could not be determined due to reaction between TNBal and compound concerned (see text).

or picric acid[26]) to partition into the mitochondrial lipid phase and inhibit phosphorylation although, like these and other strongly acidic nitro compounds, it binds to albumin amino groups. A mononitro derivative is sufficiently acidic and sufficiently lipophilic to exhibit uncoupling activity. Introduction of a 4-acyl substituent to give an exocyclic β-dione considerably potentiates the (merely marginal) uncoupling activity of phenylmethylpyrazolone. A less acidic and more lipophilic 4-acyl derivative than the 4-acetyl compound might be of interest as a potential anti-inflammatory drug.

Unfortunately, the albumin binding of 4-methylene pyrazolones (and also indan-1,3-dione itself and diphenyl-PDO, G. 14744) could not be determined using the trinitrobenzaldehyde (TNBal) reagent. Trinitrobenzaldehyde reacts directly with the activated methylene groups in these compounds and these chromogenic Claisen-type reactions are actually catalysed by albumin. N-acetylation of bovine plasma albumin much diminished, but did not abolish, the catalytic activity suggesting that both the trinitrobenzaldehyde and these acidic pyrazolones might be bound at the same or contiguous sites on the albumin—either at, or close to, certain lysyl amino groups.

The exceptional behaviour of sulphinpyrazone in uncoupling oxidative phosphorylation and exhibiting some anti-inflammatory activity, yet failing to inhibit the TNBal–albumin reaction, is all the more remarkable as it provides the only example known so far, of an acidic anti-inflammatory drug, where these properties do not run parallel.[16] However, sulphinpyrazone (1 mM) does abolish the stimulation of the TNBal–albumin reaction by 1 mM sulphamethazine (2-sulphanilamido-4,6-dimethylpyrimidine, pK 7·4)—see Table 3. If the stimulation of this reaction by sulphamethazine is due to "opening-up" of the albumin molecule when sulphamethazine is bound to it, thereby exposing lysyl groups for reaction with TNBal, then it suggests that sulphinpyrazone preferentially binds at (or near) the sulphamethazine-binding site rather than at those lysyl amino groups which normally either bind phenylbutazone or react with TNBal. Several other acidic sulphonamides either do not inhibit the TNBal–albumin reaction (e.g. sulphonacetamide, pK 5·4; sulphadiazine, pK 6·5; sulphapyridine, pK 8·4) or like sulphathiazole (pK 7·1) are rather weak inhibitors of this reaction (Table 3), supporting this idea that sulphonamides (and sulphinpyrazone) are preferentially bound at other sites on the albumin molecule than those which principally bind anti-inflammatory acids. Sulphinpyrazine is more potent than phenylbutazone in displacing albumin-bound sulphonamides.[27]

Amidopyrine, an anti-inflammatory pyrazolone,[1] does not bind appreciably to serum proteins (only 15 per cent)[28] or inhibit the TNBal–albumin reaction.[16] Some acidic potential metabolites of this drug were examined for possible uncoupling activity but only rubazo(n)icacid proved to be a potent inhibitor of mitochondrial phosphorylation.[29] Other acidic derivatives examined such as 4-hydroxyphenazone and 1-phenyl-2-methyl(and 1-phenyl-) pyrazol-5-one-3-carboxylic acid(s) did not uncouple oxidative phosphorylation when tested at 2·5 mM.

In conclusion, certain more acidic and more lipophilic analogues of phenylbutazone are more potent drugs *in vitro* than phenylbutazone (e.g. *p.p.'*-dichloro derivative or 4-α-butyryl analogue) but their poorer solubility in aqueous media may detract from their possible value as oral drugs.[30] Certain acidic pyrazolones offer some promise as drugs with uncoupling activity.

Effect of diones on papain

Ninhydrin inhibits papain,[33] an enzyme with a thiol group at its active centre.[34] The effect of hydrindantin and other diones on this enzyme was therefore examined to compare their thiol-neutralizing activity with that of ninhydrin. In order to have an indication of the rapidity with which these drugs reacted with the enzyme thiol, the esterase activity of papain[35] was utilized to assay the amount of free enzyme. Ester hydrolysis was followed by continuous titration of liberated protons.

The initial rate of hydrolysis of N-benzoyl-L-arginine ethyl ester (BAEE) was 8–10 μmoles/min, calculated for a papain concentration of 1 mg/ml, at pH 6·5 and 24° with an initial thiol (mercaptoethanol) concentration of 0·25 mM and actual enzyme concentration of 16 μg/ml. It was essential to pre-activate the enzyme with a thiol to obtain any esterase activity. Under the conditions given in the Experimental Section, the enzyme was stable for at least 3 hr and showed almost constant reaction rates of ester hydrolysis throughout this time interval.

The effects of some, 1,2-, 1,3- and 1,4-diones on this esterase activity are shown in Table 6. Hydrindantin was more potent than ninhydrin in inhibiting the enzyme even

TABLE 6. EFFECT OF SOME DIONES ON THE ESTERASE ACTIVITY OF CRYSTALLINE PAPAIN

Dione	Conc. μM	Relative reaction rates					
		A	B				(min)
			1	2	3	4	
None	—	1·0	1·0	1·0	0·93		
Ninhydrin	125	0·12	0·33	0·18	0·10		
	50	0·35	0·54	0·43	0·25		
Hydrindantin	125	0·0	0·0				
	50	0·0	0·37	0·21	0·10		
Isatin	1000	0·96	1·06	1·00	0·89		
1,2-Napthoquinone	125	0·0	0·23	0·09	0·0		
	50	0·0	0·36	0·24	0·16		
Menadione	125	0·0	0·65	0·62	0·38	0·32	
	50	0·0	0·70	0·64	0·59	0·41	
Indan-1,3-dione	2000	1·05	1·1	1·05	0·97		
2-Phenyl-ID	125	0·43	0·92	1·03	1·02		
Sulphinpyrazone	100	0·0	0·55	0·37	0·27		
Phenylbutazone	5·0	0·07	0·16	0·14			
	2·5	0·43	0·73	0·72	0·69		
Oxyphenbutazone	5·0	0·0	0·57	0·61	0·59		
	2·5	0·26	0·79	0·77	0·75		
4-Acetyl-1-Phenyl-3-methyl-PO	500	0·92	0·87	0·93	0·91		
2-Isovaleryl-ID	500	0·0	0·53	0·50	0·53		
	250	0·0	0·77	0·71	0·62		
2-Benzoyl-ID	500	0·0	0·61	0·55	0·51		
	250	0·0	0·75	0·70	0·67		
Barbituric acid	1000	0·8	1·0	0·98	0·96		
Alloxan	10	0·28	0·55	0·38	0·32	0·26	
1,3-Diphenyl-propan-1,2,3-trione	200	0·29	0·73	0·36	0·36		
	50	0·63	0·81	0·65	0·62		
1,2-Diphenyl-PDO	500	0·87	0·87	0·83	0·81		
1-Phenyl-4n.-nutyl-PDO	50	0·35	0·75	0·60	0·43		

Enzyme first preincubated with 25 mM mercaptoethanol and 20 mM Na_2 EDTA, then diluted 100-fold and pre-incubated with dione (thiol concentration = 250 μM) before adding 2 mM BAEE to start the enzyme reaction. Rate A = reaction rate for 1st 3 min after addition substrate; Rates B = enzyme reaction rates after a further addition of enzyme (80 μg) over the subsequent minute intervals (indication of the rate of inactivation of "fresh" enzyme). Respective reaction rates in absence of dione = 1·00. (Absolute rate B = 1·45–1·55 times rate A, in absence of dione).

ID = indane-1,3-dione.

PDO and PO as in Table 5.

when there was a ten-fold excess of mercaptoethanol present (relative to dione) to competitively protect the active thiol group of the enzyme. The vicinal triones, ninhydrin and alloxan, were potent inhibitors of papain but the corresponding β-diones (indan-1,3-dione and barbituric acid respectively) and isatin, a 1,2-dione analogue of ninhydrin, had little thiol-neutralizing (i.e. anti-papain) activity. Phenylbutazone (PB), and oxyphenbutazone were particularly potent inhibitors of papain even in the presence of excess thiol. Desbutyl-PB (G. 14744) did not inhibit papain under these conditions but monodesphenyl-PB (G. 29682) and sulphinpyrazone had some anti-papain activity, being approximately one-tenth as potent as PB itself. With the notable exception of phenylbutazone, other β-diones examined were generally less effective than 1,2- or 1,4-diones as inhibitors of papain esterase.

These findings suggest that sulphinpyrazone may uncouple phosphorylation because it mimics alloxan, ninhydrin and 1,2-naphthoquinone in reacting with a reactive thiol group in phosphorylating mitochondria. If so, this could also explain why it does not inhibit the TNBal–albumin reaction (like these 1,2-diones, see previous section) because it does not preferentially bind to lysyl amino groups (and therefore might inhibit mitochondrial phosphorylation by some other mechanism than associating with an essential amino group).

Two non-dione anti-inflammatory drugs which are more potent in animal assays than phenylbutazone, namely flufenamic acid and indomethacin, and which also uncouple oxidative phosphorylation,[4,36] did not affect BAEE hydrolysis by papain when tested at 1 mM. It is therefore unlikely that the anti-inflammatory acidic diones inhibit the esterase action of papain merely by "neutralizing" the arginine group of the substrate since other acidic anti-inflammatory drugs able to bind to arginine residues (see Ref. 16) do not exhibit anti-papain (esterase) properties. (Diphenadione does, however, precipitate BAEE from isotonic salt solutions). Furthermore, increasing the substrate concentration did not reverse the inhibition by diones.

GENERAL DISCUSSION

These studies suggest that 1,2- and 1,4-diones such as ninhydrin, alloxan and certain naphthoquinones which are only weak acids, or non-acidic, but react with protein thiols[33,37,38] and will inhibit papain, probably uncouple oxidative phosphorylation by reaction with a key thiol group involved in energy conservation. The uncoupling action of carbonylcyanide phenylhydrazones and 1,1,3-tricyano-2-aminoprop-1-ene is reversed by aminothiols such as cysteine and cysteamine[39] indicating that they too may owe their uncoupling activity, in part at least, to interactions with a mitochondrial thiol. The uncoupling properties of some 1,3-diones such as phenylbutazone and phenindione might also be attributable to the fact that in addition to neutralizing a reactive lysyl amino group (demonstrated by inhibition of the TNBal–albumin reaction), they may also block an active thiol group. Other uncoupling agents, such as cadmium ions and arsenicals,[40] ethacrynic acid,[41] or gold and bismuth preparations used an antirheumatic drugs,[42] would likewise appear to be bifunctional in being able to combine with both ε-amino and thiol groups.

At least one enzyme involved in energy conservation, ATP-creatine transphorylase (creatine kinase), contains both reactive (lysyl) amino[43] and thiol[44] groups which are essential for enzyme activity. Another enzyme involved in energy transfer, myosin A, has reactive amino and thiol groups which are so related that substitution of one

prevents substitution of the other.[45] If similarly disposed amino and thiol groups participate in mitochondrial ATP biosynthesis, then it is not difficult to see how drugs which can penetrate into the mitochondrial lipid phase and then react with, or block, one or both of these groups, might inhibit ATP formation.

Many thiol-blocking reagents inhibit mitochondrial respiration.[46] Interactions of diones with certain mitochondrial thiol groups could also explain why many uncoupling β-diones and related compounds (e.g. ethacrynic acid,[41] dimedones,[5] fluorodiones and fluorodihydroxybenzenes[47]) inhibit mitochondrial respiration, often at concentrations not much greater than those required to inhibit coupled phosphorylation.

2-Acyl-indandiones would appear promising as potential anti-inflammatory drugs provided that some of them can be obtained with minimal anticoagulant activity yet retaining uncoupling activity. Their ability to inhibit glycolytic phosphorylation in blow-fly sarcosomes[48] and bacterial properties[49] have been briefly reported.

Acknowledgements—Financial support was provided by the Nuffield Foundation, London, the Department of Education of Science and J. R. Geigy, (U.K.) Ltd., Manchester. We are also much indebted to Dr. R. Pfister, Dr. P. B. Fowler and other donors of compounds, and to Mr. R. L. J. Gardener for technical assistance with some of these experiments.

REFERENCES

1. R. DOMENJOZ, *Ann. N.Y. Acad. Sci.* **86**, 263 (1960).
2. L. FONTAINE, M. GRAND, Y. QUENTIN and S. MERLE, *Med. Pharmac. exp.* **13**, 137 (1965).
3. S. S. ADAMS and R. COBB, *Nature, Lond.* **181**, 773 (1958).
4. M. W. WHITEHOUSE and J. M. HASLAM, *Nature, Lond.* **196**, 1323 (1962).
5. I. F. SKIDMORE and M. W. WHITEHOUSE, *Biochem. Pharmac.* **14**, 547 (1965).
6. M. W. WHITEHOUSE, *Forschr. Arzneimitt Forsch.* **8**, 321 (1965).
7. A. W. DOX, in *Org. Syntheses*, (Ed. H. GILMAN), collective vol. I, p. 261 (1932).
8. L. B. KILGORE, J. H. FORD and W. C. WOLFE, *Ind. Engng Chem.* **34**, 494 (1942).
9. R. L. HORTON and K. C. MURDOCK, *J. org. Chem.* **25**, 938, (1960).
10. N. J. CARTWRIGHT, J. IDRIS JONES and D. MARMION, *J. chem. Soc.* 3499 (1952).
11. A. SCHÖNBERG and R. C. AZZAM, *J. chem. Soc.* 1428 (1939).
12. W. O. TEETERS and R. L. SHRINER, *J. Am. chem. Soc.* **55**, 3026 (1933).
13. B. GRAHAM, U.S. Pat. 2,694,703 (1954); (*Chem. Abstr.* **49**, 3706).
14. R. A. DARROW and S. P. COLOWICK, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. KAPLAN), vol. V, p. 226. Academic Press, New York (1962).
15. I. F. SKIDMORE and M. W. WHITEHOUSE, *J. Pharm. Pharmac.* **17**, 671 (1965).
16. I. F. SKIDMORE and M. W. WHITEHOUSE, *Biochem. Pharmac.* In press.
17. M. W. WHITEHOUSE and P. D. G. DEAN, *Biochem. Pharmac.* **14**, 557 (1965).
18. S. RUHEMANN, *J. chem. Soc.* **792**, 1306 (1911).
19. F. BÖHM, *Z. physiol. Chem.* **269**, 24 (1941).
20. A. K. VLČEK, E. ŠPALCK and L. KRÁTKÝ, *Colln Czech. chem. Commun.* **15**, 340 (1950).
21. E. YOUNATHAN, *Arch. Biochem. Biophys.* **113**, 439 (1966).
22. M. GRIFFITHS, *Arch. Biochem. Biophys.* **20**, 451 (1949).
23. D. D. VAN SLYKE, R. T. DILLON, D. A. MACFADYEN and P. HAMILTON, *J. biol. Chem.* **141**, 627 (1941).
24. E. CHARGAFF and A. BENDICH, *J. biol. Chem.* **149**, 93 (1943).
25. R. D. DALLAM and J. W. HAMILTON, *Arch. Biochem. Biophys.* **105**, 630 (1964).
26. J. E. LEADER and M. W. WHITEHOUSE, *Biochem. Pharmac.* **15**, 1379. (1966).
27. A. H. ANTON, *J. Pharmacol.* **134**, 291 (1961).
28. B. B. BRODIE and J. AXELROD, *J. Pharmacol.* **99**, 171 (1950).
29. G. M. SMITH, M. E. PARSONS and M. W. WHITEHOUSE, *J. Pharm. Pharmac.* **16**, 830 (1964).

30. B. B. BRODIE, in *Absorption and Distribution of Drugs* (Ed. T. B. BINNS), p. 45. Livingstone, Edinburgh, (1964).
31. P. E. GAGNON, J. I. BOIVIN and R. J. PAQUIN, *Can. J. Chem.* **31**, 1025 (1953).
32. J. M. PEREL, M. M. SNELL, W. CHEN and P. G. DAYTON, *Biochem. Pharmac.* **13**, 1305 (1964).
33. F. TAYEAU, J. MARCQUEVILLE and S. MARCQUEVILLE-REGNIER, *Bull. Soc. Pharm. Bordeaux.* **92**, 239 (1954), *Chem. Abstr.* **50**, 6530 (1956).
34. E. L. SMITH and J. R. KIMMEL, in *The Enzymes* 2nd Ed. (Eds. P. D. BOYER, H. LARDY and K. MYRBÄCK) vol. 4, p. 134. Academic Press, New York (1960).
35. C. E. MCDONALD and A. K. BALLS, *J. biol. Chem.* **229**, 69 (1957).
36. M. W. WHITEHOUSE, *Nature, Lond.* **201**, 629. (1964).
37. J. L. WEBB, *Enzyme and Metabolic Inhibitors*, vol. III, pp. 367, 421, Academic Press, New York (1966).
38. D. YU-CHANG and T. CHEN-LU cited by M. ERRERA, *Nature, Lond.* **205**, 739 (1965).
39. P. G. HEYTLER, *Biochemistry* **2**, 357 (1963).
40. A. L. FLUHARTY and D. R. SANADI, *Biochemistry* **2**, 519 (1963).
41. Y. GAUDEMER, B. FOUCHER and D. GAUTHERON, *C.r. hebd. Séanc Acad. Sci., Paris* **261**, 3899 (1965).
42. M. W. WHITEHOUSE, *Biochem. J.* **92**, 36P (1964).
43. H. G. JACOBS and L. W. CUNNINGHAM, *Fedn Proc.* **25**, 407 (1966).
44. T. A. MAHOWALD, *Biochemistry* **4**, 732 (1965).
45. S. KUBO, S. TOKURA and Y. TONOMURA, *J. biol. Chem.* **235**, 2835 (1960).
46. W. S. LYNN, JR. and R. H. BROWN, *Biochim. Biophys. Acta* **110**, 445 (1965).
47. M. W. WHITEHOUSE and I. F. SKIDMORE, *Biochem. Pharmac.* In press (1967).
48. J. W. RAY, *Biochem. J.* **95**, 40P (1965).
49. C. H. HASSALL, *Experientia*, **6**, 462 (1950).