Biochemical Pharmacology, Vol. 19, pp. 759–768. Pergamon Press. 1970. Printed in Great Britain

THE INHIBITION OF DRUG METABOLISM ENZYMES BY SOME NATURALLY OCCURRING COMPOUNDS

PENELOPE S. GRAHAM, R. O. HELLYER* and A. J. RYAN

Department of Pharmacy, The University of Sydney, Sydney, N.S.W., 2006, Australia

(*Received* 12 *February* 1969; *accepted* 9 *September* 1969)

Abstract—The ability of a series of naturally occurring pyrethrin synergists to inhibit various pathways of drug metabolism has been studied in the rat and mouse. These compounds were effective *in vitro* inhibitors of the oxidative metabolism of aniline, aminopyrine, diphenyl and hexobarbital by liver preparations. Reduction of the azo dye neoprontosil was not inhibited. *In vivo* inhibition of hexobarbital oxidation in the mouse, as shown by an increase in sleeping times, was also observed. Some considerations of structure–activity relationships are discussed. Several of the compounds studied produced dilation of the endoplasmic reticulum and the Golgi apparatus of liver cells as well as depleting glycogen deposits. These effects did not appear to be related to inhibitory power. These substances can alter membrane permeability, as shown by measurements of glucose exit from red blood cells. The effects on the liver and red blood cell may be a function of lipid solubility or, more likely, ability to bind to protein.

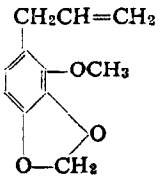
A NUMBER of compounds which synergise insecticides inhibit the metabolism of drugs and insecticides by both animals[1] and insects.[2] The most active synergists which are used commercially contain a methylenedioxyphenyl moiety.[3] The reasons for this are not known with certainty but a number of suggestions have been advanced.[4, 5] At present it appears that the synergist competes with the insecticide for the enzyme responsible for detoxication of the insecticide.[6]

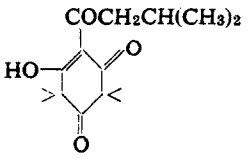
A number of compounds (Fig. 1) occurring in Australian flora have been found to act as pyrethrin synergists when tested against the housefly *Musca domestica*.[7] Since most of these are closely related structurally they provided an opportunity to examine structure–activity relationships in their effects on the drug metabolising enzymes of animals. The present report is concerned with the study of such effects both *in vivo* and *in vitro* on oxidative and reducing systems in the rat and mouse. The inhibitory activities of these compounds have been compared with SKF-525A, piperonyl butoxide, phloretin and phlorizin. In addition the effects of some of these compounds on the ultrastructure of liver cells and the permeability of red blood cells are reported.

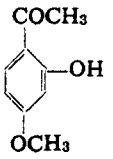
MATERIALS AND METHODS

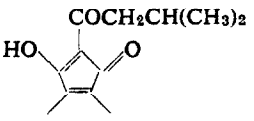
The inhibitors (Fig. 1) were obtained from the Museum of Applied Arts and Sciences, Sydney, Australia. SKF-525A (β-diethylaminoethyl-diphenylpropyl acetate hydrochloride) was donated by Smith, Kline and French Laboratories, Australia. Technical grade piperonyl butoxide (87–90% pure) was the gift of William Cooper

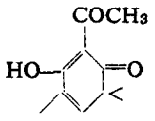
* Present address: Riker Laboratories Australia Pty. Ltd., Thornleigh, N.S.W., 2120, Australia.

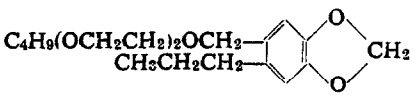

Croweacin

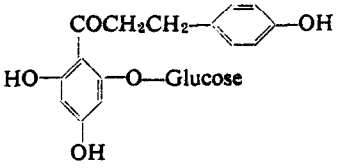

Leptospermone

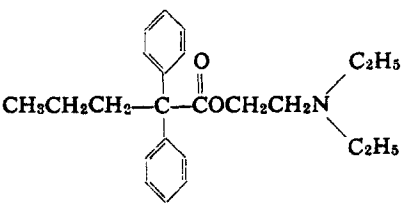

Paeonol

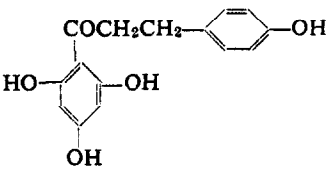

Calythrone

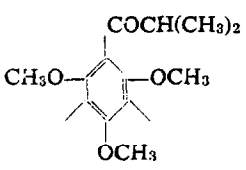

Dehydroangustione

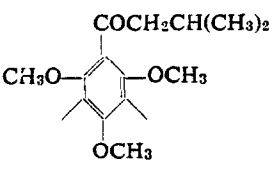

Piperonyl butoxide

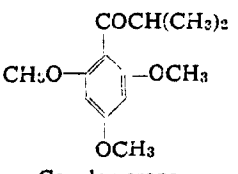

Phlorizin

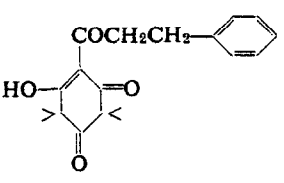

SKF 525A

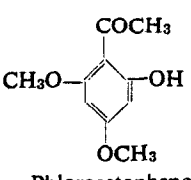

Phloretin

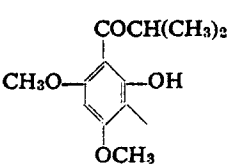

Camfieldione

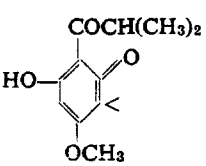

Torquatone

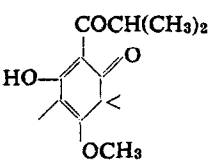

Conglomerone


Grandiflorone


Phloracetophenone dimethyl ether


Baeckeol


Agglomerone


Tasmanone

and Nephews (Aust.) Pty. Ltd. Phlorizin was obtained from British Drug Houses Ltd., England, and the aglycone, phloretin, prepared from phlorizin by acid hydrolysis according to Miller and Robertson.[8] NADP and glucose-6-phosphate were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

In vivo *studies*

Adult male white mice were used to determine the duration of hexobarbital hypnosis. The compounds were dissolved in arachis oil and administered intraperitoneally, 1 hr before the hexobarbital unless otherwise stated. Control mice were injected with an equivalent volume of arachis oil. During hypnosis, mice were kept in an incubator set at 35–36°. In each experiment ten animals were dosed and ten used as controls.

In vitro *studies*

Male albino Wistar strain rats (70–80 g), or adult male mice were killed by cervical dislocation. Livers were perfused with isotonic potassium chloride (0°), removed, and a 25% homogenate in 0·15 M phosphate buffer (pH 7·4) prepared. The homogenate was centrifuged at 9000 *g* for 20 min and the supernatant used in enzyme assays. The oxidative metabolism of aminopyrine, aniline, diphenyl and hexobarbital were studied, all incubations being carried out at 37°. All control incubations with substrate only showed a linear time course for 30 min. 4-Aminoantipyrine, *p*-aminophenol and hexobarbital were assayed according to Gilbert and Golberg.[9] With minor modifications the fluorometric estimation of diphenyl metabolites was according to Creaven *et al.*[10] using an Aminco–Bowman spectrofluorometer. Sulphanilamide, a metabolite of neoprontosil, was assayed according to Bratton and Marshall.[11] The coupled product was extracted into isoamyl alcohol to separate it from unmetabolised neoprontosil.[12] The incubation medium consisted of liver homogenate (2 ml), NADP (0·15 μmole), glucose-6-phosphate (10 μmoles), nicotinamide (50 μmoles), magnesium sulphate (25 μmoles), substrate and inhibitor, to a total volume of 3 ml. The final buffer concentration was 0·1 M. For aniline hydroxylation the medium contained NADP (0·75 μmole), glucose-6-phosphate (18 μmoles), nicotinamide (75 μmoles), magnesium sulphate (30 μmoles), as well as inhibitor, substrate and homogenate in a total volume of 3 ml. Thunberg tubes, evacuated, were used for assays of azoreductase activity, where the pH was reduced to 7·2. Where necessary, substrate or inhibitors were solubilised in Tween 80, the final concentration being 0·1%. Control flasks contained equivalent concentrations of Tween 80.

Electron microscopy

The inhibitors were administered intraperitoneally to 70–80 g male rats. After 1 hr they were killed and sections of liver removed, fixed in osmium tetroxide and embedded in Araldite. A lead hydroxide stain was used.

Glucose transport across the red blood cell membrane

A method similar to that of LeFevre and Marshall[13] was used to estimate the rate of exit of glucose from red blood cells. Fresh heparinised human blood was centrifuged to isolate the erythrocytes, which were washed several times with an isotonic buffer (pH 7·4) prepared according to LeFevre and Marshall.[13] The cells were incubated for 1 hr at 37° in a solution containing 2·52% glucose and 0·63% sodium chloride.

Aliquots were then pipetted into a solution of the inhibitor in the isotonic buffer; the final cell concentration being 0·5%. The subsequent change in absorbance measured resulted from shrinkage and crenation of the red blood cells as glucose was released. The rate of change of absorbance is a measure of the rate of exit of glucose from the red blood cells. An EEL Colorimeter with red filter was used to measure absorbance.

RESULTS

The sleeping times of mice dosed with hexobarbital 1 hr after dosage with some of the inhibitors is shown in Table 1. Of the naturally occurring substances only camfieldione was comparable with piperonyl butoxide or SKF-525A. The remainder were only weakly active or inactive. At 50 mg/kg camfieldione was effective up to 8 hr after dosing (Fig. 2).

TABLE 1. HEXOBARBITAL SLEEPING TIMES IN MICE PRETREATED FOR 1hr WITH INHIBITORS

Compound	Dose mg/kg	Duration of sleep ± S.D. (min)	Percent of control	P
Control	—	28 ± 8	100	—
SKF-525A	20	88 ± 20	320	0·01
Piperonyl	50	59 ± 24	214	,,
butoxide	100	126 ± 13	455	,,
Camfieldione	50	81 ± 10	294	,,
	100	100 ± 23	362	,,
Conglomerone	50	42 ± 14	152	,,
	100	59 ± 12	214	,,
Grandiflorone	100	45 ± 13	164	,,
Tasmanone	50	27 ± 5	98	>0·05
	100	30 ± 8	109	,,
Leptospermone	100	29 ± 8	105	,,
Phloretin	50	30 ± 6	109	,,
	100	29 ± 7	105	,,
Phlorizin	50	29 ± 8	105	,,
	200	31 ± 8	114	,,

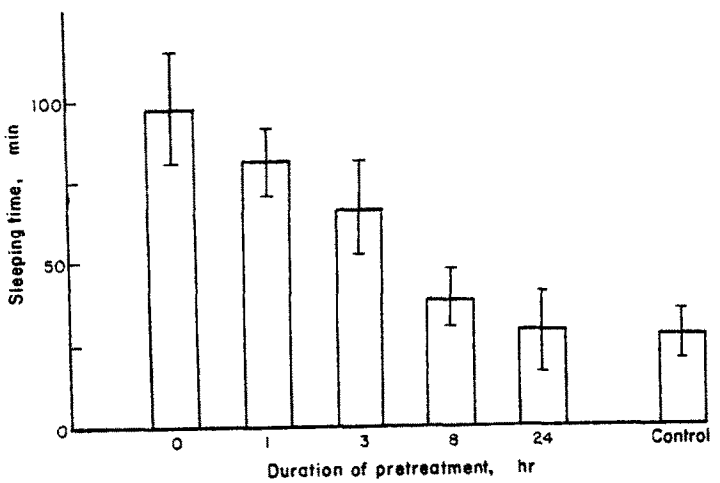


FIG. 2. The effect on hexobarbital sleeping time of camfieldione (50 mg/kg) administered at various times before the hexobartital (both by intraperitoneal route).
The range of ± one standard deviation is shown.

Inhibition in vitro

Demethylation of aminopyrine, *para*-hydroxylation of diphenyl and aniline, and side chain oxidation of hexobarbital were inhibited to varying degrees by the synergists tested. The relative degrees of inhibition of aminopyrine demethylation by the synergists were similar in both rat (Table 2) and mouse liver preparations (Table 3). There appeared to be no significant inhibitory effect on the *ortho*-hydroxylation of diphenyl although the accurate measurement of this isomer in the presence of a much larger quantity of *p*-OH diphenyl was difficult. These results are not given. In addition

TABLE 2. THE INHIBITION OF THE METABOLISM OF VARIOUS SUBSTRATES *IN VITRO* BY RAT LIVER

Inhibitor	Substrate			
	Aminopyrine	Diphenyl	Aniline	Hexobarbital
Camfieldione	6 ± 2	64 ± 10	82 ± 4	—
Torquatone	8 ± 2	60 ± 4	—	60 ± 11
Conglomerone	18 ± 4	48 ± 5	—	—
Grandiflorone	20 ± 2	36 ± 3	55 ± 2	—
Phloracetophenone dimethyl ether	26 ± 4	—	—	—
Baeckeol	27 ± 2	65 ± 6	—	—
Agglomerone	30 ± 13	—	—	—
Tasmanone	34 ± 2	42 ± 3	77 ± 1	—
Croweacin	38 ± 4	—	—	—
Leptospermone	42 ± 6	46 ± 2	61 ± 3	45 ± 9
Paeonol	54 ± 1	—	—	—
Calythrone	63 ± 3	—	—	—
Dehydroangustione	80 ± 1	58 ± 3	—	—
Piperonyl Butoxide	12 ± 1	85 ± 4	41 ± 3	40 ± 15
Phlorizin	86 ± 5	—	—	—
Phloretin	28 ± 4	—	—	—
SKF 525A				
1×10^{4} M)	—	68 ± 3	—	30 ± 10
2×10^{-4} M)	7 ± 1	65 ± 2	—	—
4×10^{-4} M)	—	32 ± 5	50 ± 3	—

Inhibitor concentration was 4×10^{-4} M unless otherwise stated. Incubations were carried out at 37°.

Amino pyrine and hexobarbital concentrations were $6{\cdot}7 \times 10^{-4}$ M, aniline and diphenyl concentrations were 2×10^{-3} M.

Results are expressed as per cent of control.

TABLE 3. INHIBITION OF AMINOPYRINE DEMETHYLATION *IN VITRO* IN MOUSE LIVER

Inhibitor	Amount metabolised
	% of control
Conglomerone	11 ± 3
Grandiflorone	37 ± 2
Agglomerone	49 ± 3
Tasmanone	67 ± 3
Leptospermone	85 ± 4
Calythrone	103 ± 5
Dehydroangustione	85 ± 3

Substrate concentration $6{\cdot}7 \times 10^{-4}$ M.
Inhibitor concentration 4×10^{-4} M.
Incubations were carried out at 37°.

TABLE 4. THE EFFECT OF SIX INHIBITORS OF OXIDATIVE DRUG METABOLISM ON THE REDUCTION OF NEOPRONTOSIL

Inhibitor	Percent of control	
	Neoprontosil $3{\cdot}3 \times 10^{-3}$ M	Neoprontosil $3{\cdot}3 \times 10^{-4}$ M
Camfieldione	90	93
Agglomerone	105	120
Leptospermone	94	109
Dehydroangustione	100	110
Piperonyl butoxide	103	109
SKF-525A	117	109

Inhibitor concentration, 4×10^{-4} M.
Incubation was carried out under anaerobic conditions for 15 min at 37°. Sulphanilamide liberated was assayed.

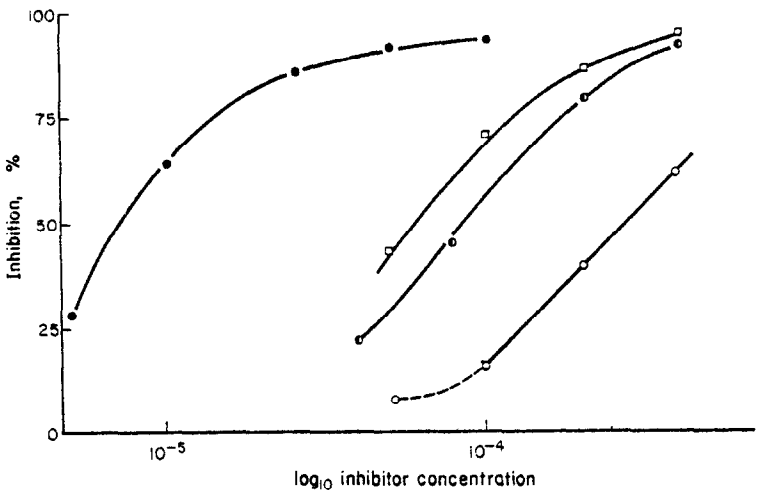


FIG. 3. The relationship between log 10 inhibitor concentration and percent inhibition for 4 inhibitors.
(●) SKF-525A (◑) Piperonyl Butoxide
(□) Camfieldione (○) Baeckeol

there was no effective inhibition of azo reductase activity irrespective of substrate concentration (Table 4).

The log-dose–response curve for the inhibition by four compounds is given in Fig. 3. SKF-525A is at least ten times more potent than the insecticide synergists; the similarity in slope of the curves suggest that the inhibitors are acting in a similar manner.[14]

Electron microscopy

The effects on liver cells of the six compounds tested are summarised in Table 5. Grandiflorone and SKF-525A caused much vacuolation of the rough surfaced endoplasmic reticulum. The degree of vacuolation varied depending on the test compound. Depletion of glycogen deposits and enlargement of the Golgi apparatus were frequently observed; in some cases other indications of incipient liver damage[15, 16] were observed.

TABLE 5. THE EFFECT OF INHIBITORS OF DRUG METABOLISM ON THE INTRACELLULAR MORPHOLOGY OF THE HEPATIC CELL

Compounds	Dose (mg/kg)	Endoplasmic reticulum	Golgi Apparatus	Glycogen depletion
Grandiflorone	100	+++++	+	+
SKF-525A	50	++++	+	+
Phloretin	100	++++	+	+
Conglomerone	50	++	−	−
Piperonyl butoxide	50	++	?	−
	100	++	+	±
Phlorizin	160	+	++	?

The degree of vacuolation of the rough endoplasmic reticulum is indicated by the number of crosses. Enlargement of the Golgi Apparatus and apparent depletion of glycogen deposits are also indicated by crosses. The inhibitors are listed in decreasing order of potency, estimated by effect on the microsomal membranes.

TABLE 6. THE EFFECT OF SOME INHIBITORS OF DRUG METABOLISM ON EXIT OF GLUCOSE FROM RED BLOOD CELLS

Inhibitor	Concentration ($\times 10^{-4}$ M)	Percent change in absorbance
Nil. (Control)	—	37
Phloretin	3·3	0
	0·4	3
Baeckeol	3·8	3
	0·8	22
SKF-525A	3·3	5
	0·8	25
Camfieldione	3·3	6
Torquatone	3·3	14
Phloracetophenone dimethyl ether	3·3	16
Piperonyl butoxide	3·3	21
Grandiflorone	3·3	22
Tasmanone	3·3	22
Conglomerone	3·3	26
Leptospermone	3·3	28
Dehydroangustione	3·3	32

The rate of exit of glucose is proportional to the percent change in absorbance during the first 3 min of incubation. Theinhibitors are listed in decreasing order of ability to inhibit glucose transport.

Glucose transport across the red blood cell membrane

At a concentration similar to that used in *in vitro* studies of inhibition of aminopyrine demethylation, all the inhibitors delayed the exit of glucose from the red blood cell (Table 6). The percent change in absorbance after 3 min is proportional to the rate of glucose exit. Phloretin was considerably more active than the insecticide synergists.

DISCUSSION

An examination of the structures of the compounds tested as inhibitors of hexobarbital metabolism *in vivo* indicates that those compounds which would first have to

undergo oxidative metabolism prior to conjugation and excretion were more active than those which could be readily conjugated. Whereas grandiflorone and piperonyl butoxide could not be conjugated until they had been hydroxylated, tasmanone and phloretin, which possess free hydroxyl groups, may well be rapidly conjugated and hence inactivated soon after administration. Camfieldione and conglomerone would possibly be demethylated prior to conjugation. Both piperonyl butoxide[6] and SKF-525A[17] are known to undergo oxidative metabolism in mammalian systems. Phlorizin showed no ability to inhibit oxidative drug metabolism *in vivo*, and had only a mild inhibitory effect *in vitro*, possibly due to the presence of small amounts of phloretin. The inability of phlorizin to inhibit drug metabolism is presumably a result of its low lipid solubility and, *in vivo*, due to the rapidity with which it is concentrated in the kidneys and excreted.[18]

In vitro, glucuronide conjugation will not occur readily unless UDP-glucuronic acid is added to the incubation medium[19] hence a wider range of compounds were observed to inhibit drug metabolism *in vitro*. Phloretin, inactive *in vivo*, was as potent an inhibitor of aminopyrine demethylation as grandiflorone *in vitro*. The relative activities of the synergists as inhibitors of drug metabolism varied, depending on the pathway studied.

The relationship between structure and inhibitory activity *in vitro* is not immediately obvious. Recent work (Ryan and Graham, unpublished) has provided some indications of the structural requirements for activity in the ketone series. This seems to be a planar arrangement of a carbonyl group attached to a flat ring with hydroxyl groups flanking the carbonyl function. In accord with this, 2,6-dihydroxyacetophenone is a very good inhibitor of drug metabolism *in vitro*. Acetophenone and *o*-hydroxyacetophenone are not nearly as effective. These results will be elaborated in a later publication on structural effects in this series.

Irrespective of substrate concentration, there was no inhibition of azo reductase activity by the compounds studied. This is not surprising if it is assumed that azo-compounds are reduced by NADPH-cytochrome *c*-reductase.[20] Mazel and Herandez[21] have, however, shown that cytochrome P-450 may also be involved in azo-reduction. SKF-525A is known to be a weak inhibitor of azo reductase,[22] and may be inhibiting the P-450 dependent pathway.

There was no strict correlation between inhibition of drug metabolism *in vivo* and the degree of effect on the ultra structure of the liver cell. Phloretin (100 mg/kg) had as marked an effect as SKF-525A (50 mg/kg) on the endoplasmic reticulum, but had no effect on hexobarbital metabolism at the same dose level. Although piperonyl butoxide caused a marked increase in hexobarbital sleeping time, only mild dilation of the endoplasmic reticulum was observed. These results indicate that swelling of the rough-surfaced endoplasmic reticulum is not a measure of the inhibition of drug metabolism. The fact that a number of drugs and hepatotoxic agents,[15, 23] as well as insecticide synergists cause both dilation of the membranes and inhibition of drug metabolism suggests that these two effects are independently caused by the presence of foreign compounds in the liver. The dependence on the presence of intact phospholipid for the structural integrity of microsomal P-450[24] may indicate a relationship between effect on the microsomal membranes and inhibition of drug metabolism. Both rough and smooth surfaced microsomal membranes contain the enzyme system capable of oxidising foreign compounds,[25] and there is evidence that these two

membrane types are continuous within the cell.[26] Dilation of the endoplasmic reticulum may be a function of the lipid solubility of a foreign compound, or of its ability to bind to protein, and may therefore be a response to the presence of foreign compounds, possibly enabling more rapid removal of these compounds from the cell.[26] Proliferation of the Golgi apparatus noted in this study may be the initial step in the drug-induced increase in smooth-surfaced membranes, since continuity between the smooth endoplasmic reticulum and the Golgi apparatus has been reported.[16, 26] Proliferation of the Golgi apparatus has been observed after treatment with certain drugs and in regenerating liver.[16] Changes in the distribution of the ribosomal particles and depression of glycogen have been reported associated with conditions resulting in depressed drug metabolism.[27, 28]

Since phloretin was observed to inhibit drug metabolism *in vitro*, it was thought possible that inhibitors of drug metabolism may also inhibit glucose exit from the red blood cell. This did in fact occur. It would be inadvisable to draw too close a parallel between effects of inhibitors on the red blood cell membrane and effects noted in the liver. However it is interesting to note that the inhibitors tested caused changes in both the red blood cell membranes and the endoplasmic reticulum. One can speculate whether inhibitors of drug metabolism and hepatotoxic agents deplete glycogen levels by affecting glucose transport in the liver cell in a similar way to the red blood cell. Whether this could be due to changes in the hepatocyte plasma membrane or to the observed changes in the endoplasmic reticulum is not known.

Acknowledgements—This work was supported by grant EF 258 of the National Institute of Health, U.S. Public Health Service and the National Health and Medical Research Council of Australia.

REFERENCES

1. N. C. Leeling and J. E. Casida, *J. Agr. Food Chem.* **14**, 281 (1966).
2. W. W. Phillco, R. D. Schonbrod and L. C. Terriere, *J. Agr. Food Chem.* **13**, 113 (1965).
3. H. H. Moorefield and M. H. J. Weiden, *Cont. Boyce Thompson Inst.* **22**, 425, (1964).
4. T. Nakatsugawa and P. A. Dahm, *J. Econ. Ent.* **58**, 500 (1965).
5. D. J. Hennessy, *J. Agric. Fd. Chem.* **13**, 218 (1965).
6. J. E. Casida, J. L. Engel, E. G. Essac, F. X. Kamienski and S. Kuwatsuka, *Science* N.Y. **153**, 1130 (1966).
7. R. W. Kerr, *C.S.I.R.O. Bull.* **261**, 1 (1951).
8. A. Miller and A. Robertson, *J. Chem. Soc.*, 1170.(1933).
9. D. Gilbert and L. Golberg, *Fd. Cosmet. Toxic.* **3**, 417 (1965).
10. P. J. Creaven, D. V. Parke and R. T. Williams, *Biochem. J.* **96**, 879 (1965).
11. A. C. Bratton and E. K. Marshall Jr, *J. biol. Chem.* **128**, 537 (1939).
12. J. R. Fouts, J. J. Kamm and B. B. Brodie, *J. Pharmac. exp. Ther.* **120**, 291 (1957).
13. P. G. Le Fevre and J. K. Marshall, *J. biol. Chem.* **234**, 3022 (1959).
14. E. J. Ariens, A. M. Simones and J. M. Van Rossum, in *Medicinal Chemistry* **3** (Ed. E. J. Ariens) p. 119, Academic Press, New York (1964).
15. K. R. Rees, in *Ciba Symposium on Cellular Injury*. (Eds. A. V. S. DeReuck and J. Knight) p. 53, J. & A. Churchill, London (1964).
16. Ch. Rouiller and A. M. Jezequel, in *The Liver* **1**, (Ed. Ch. Rouiller) p. 195, Academic Press, New York (1963).
17. M. W. Anders, A. P. Alvares and G. J. Mannering, *Mol. Pharmac.* **2**, 328 (1966).
18. W. D. Lotspeich, *Harvey Lect.* **56**, 63 (1960).
19. G. H. Lathe and M. Walker, *Biochem. J.* **70**, 705 (1958).
20. P. H. Hernandez, P. Mazel and J. R. Gillette, *Pharmacologist* **7**, 147 (1965).
21. P. Mazel and P. Hernandez, *Fedn. Proc.* **26**, 461 (1967).
22. J. R. Gillette, *Prog. Drug Res.* **6**, 13 (1963).

23. M. BASSI, *Expl Cell. Res.* **20**, 313 (1960).
24. T. OMURA and R. SATO, *J. biol. Chem.* **239**, 2379 (1964).
25. T. E. GRAM, L. A. ROGERS and J. R. FOUTS, *J. Pharmac. exp. Ther.* **155**, 479 (1967).
26. K. R. PORTER, *The Cell*, Vol. 2 (Ed. J. BRACHET and A. E. MIRSKY) p. 621, Academic Press, New York (1961).
27. J. N. LOEB, R. HOWELL and G. M. TOMKINS, *J. biol. Chem.* **242**, 2069 (1967).
28. J. R. FOUTS, *Proc. 2nd Intern. Pharmac. Meeting* 1963 **4**, 261 (1965).