Biochemical Pharmacology, 1966, Vol. 15, pp. 1469–1477. Pergamon Press Ltd., Printed in Great Britain.

DIFFERENTIATION OF THE BARBITURATE STIMULATION OF THE GLUCURONIC ACID PATHWAY FROM *DE NOVO* ENZYME SYNTHESIS

E. M. AARTS

Institute of Pharmacology, University of Nijmegen, Nijmegen, The Netherlands

(*Received* 12 *April* 1966; *accepted* 17 *May* 1966)

Abstract—In order to elucidate the function of UDPG dehydrogenase (1.1.1.21) in the stimulation of the glucuronic acid pathway by barbiturate, UDPG dehydrogenase and D-glucuronolactone dehydrogenase (1.1.1.70) activities were determined in the liver of barbital-treated rats and guinea pigs. Increased urinary excretion of L-ascorbic acid and D-glucaric acid was used as a criterion for the occurrence of stimulation. UDPG dehydrogenase activity was not significantly enhanced, but excretion of the acids as well as microsomal demethylation of amidopyrine were increased.

Treatment of rats with the stress agent celite, an inducer of tyrosine aminotransferase (2.6.1.5) via adrenal hydrocortisone, had no influence on excretion of the acids or on activities of UDPG dehydrogenase, D-glucuronolactone dehydrogenase and microsomal demethylation.

A single dose of chloretone administered to rats stimulated markedly L-ascorbic acid excretion without increasing UDPG dehydrogenase activity. Chronic treatment with chloretone stimulated excretion of the acid and increased UDPG dehydrogenase activity.

Puromycin and actinomycin D did not prevent the stimulatory effect of barbital on excretion of the acids. It was concluded that the stimulation of the glucuronic acid pathway by barbiturate is not the result of an enhanced synthesis of enzymes concerned in the glucuronic acid pathway.

THE stimulatory effect of drugs on the biosynthesis of L-ascorbic acid, D-glucuronic acid, L-xylulose and D-glucaric acid[1–4] can be explained by an increased formation of the common intermediate D-glucuronic acid or its lactone[5] from UDPG (Fig. 1).

The possibility that this stimulation would be a result of an elevated level of liver enzymes concerned in the glucuronic acid pathway was suggested by the observation that ethionine, an inhibitor of protein synthesis, prevented the reaction of normal rats to the stimulating drugs.[6, 7] Available evidence now indicates that the inhibition of protein synthesis in the liver by ethionine is secondary to a decrease in the concentration of ATP.[8] The latter decrease would also explain the strong decrease in the levels of fructose-1,6-diphosphate, glucose-6-phosphate and NAD.[9] The effect of ethionine on the response to drugs could then be explained from the decreased availability of glycolytic intermediates and NAD for conversion to UDP-glucuronic acid, rather than through inhibition of enzyme synthesis.

In studies of the effect of drug administration on rat liver enzymes concerned in L-ascorbic acid synthesis, UDPG dehydrogenase (1.1.1.22) and UDP glucuronyltransferase (2.4.1.17) were found to display enhanced activities, possibly due to *de*

novo enzyme synthesis. Of drugs increasing urinary L-ascorbic acid excretion, chloretone[7, 10] and barbital[10] also stimulate UDPG dehydrogenase activity. Stimulation of UDPG dehydrogenase activity by 3,4-benzpyrene[11] was not confirmed by other investigators.[7] Carcinogenic hydrocarbons and amidopyrine had no influence on UDPG dehydrogenase activity, but enhanced UDP glucoronyltransferase activity.[7]

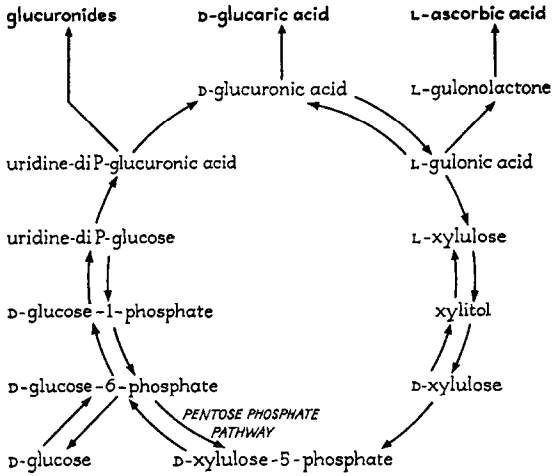

FIG. 1. The glucuronic acid pathway of glucose metabolism.

The postulate[12] that an increased level of UDP glucuronyltransferase would result in a stimulation of L-ascorbic acid biosynthesis is not supported by further observations.[3,13] Sedormide, a potent stimulator of urinary L-ascorbic acid excretion,[14] inhibits glucuronide formation *in vivo* and *in vitro*.[15] The question whether the enhanced activity of UDPG dehydrogenase, as a result of *de novo* enzyme synthesis, could be responsible for the stimulation by chloretone or barbital has not been definitively answered. The observation of de Matteïs[14] that actinomycin D did not inhibit the stimulary effect of 2-allyl-2-isopropylacetamide on L-ascorbic acid excretion by rats suggests that *de novo* enzyme synthesis is not involved in the stimulation.

This investigation has been aimed at elucidating the function of UDPG dehydrogenase in the stimulation of the glucuronic acid pathway by barbiturate. The experimental animals were rats and guinea pigs. Urinary excretion of L-ascorbic acid and D-glucaric acid was used as a criterion for the occurrence of stimulation. Liver UDPG dehydrogenase and D-glucuronolactone dehydrogenase (1.1.1.70) activities were compared with the activities of inducible enzyme systems, and the influence of puromycin and actinomycin D on the stimulation by barbiturate was studied.

MATERIALS AND METHODS

Guinea pigs were maintained on Hope Farms Laboratory Diet, with free access to water and an extra supply of 20 mg L-ascorbic acid per day. Wistar rats were maintained on L-ascorbic acid free Hope Farms Laboratory Diet with free access to water. For determination of L-ascorbic acid, urine was collected in 10% oxalic acid solutions and stored at 4°. L-Ascorbic acid was determined by the 2,6-dichlorophenolindophenol method, and D-glucaric acid by its specific inhibitory effect after acid treatment upon

β-glucuronidase.[17] Suspensions of celite (a diatomaceous earth, Johns-Manville) were homogenized to reduce particle size and facilitate injection. Chloretone was given in an emulsion by stomach tube. Puromycin (Serva) and actinomycin D (Merck Sharp and Dohme) were dissolved in buffered saline,[18] and given intraperitoneally.

Enzymic assays

Immediately after sacrifice, the livers of the animals were removed, cooled in ice, and homogenized in 4 volumes of 0·15 M KCl in a Potter-Elvehjem grinder. Homogenates were centrifuged for 30 min at 9000 *g* and the supernatants were used for all assays. UDPG dehydrogenase was determined according to Strominger *et al.*,[19] D-glucuronolactone dehydrogenase according to Marsh.[16] Demethylation activity was determined by the procedure described by Smith *et al.*[20] with slight modifications. Glucose-6-phosphate dehydrogenase (5 Kornberg units of G-6 POH 15303 Boehringer) was added, amidopyrine was used as substrate, and the incubation time was 1 hr. Tyrosine aminotransferase was determined according to Kenney,[21] except that pyridoxal phosphate was omitted during incubation. Proteins were estimated by the Lowry procedure,[22] using bovine serum albumin (Sigma) as a standard.

RESULTS

After treatment of guinea pigs with barbital a strong increase of D-glucaric acid concentration in urine was observed, but no increase of the UDPG dehydrogenase activity in the liver was detected. There was no correlation between D-glucaric acid excretion and enzyme activity (Fig. 2). Since the increased D-glucaric acid excretion

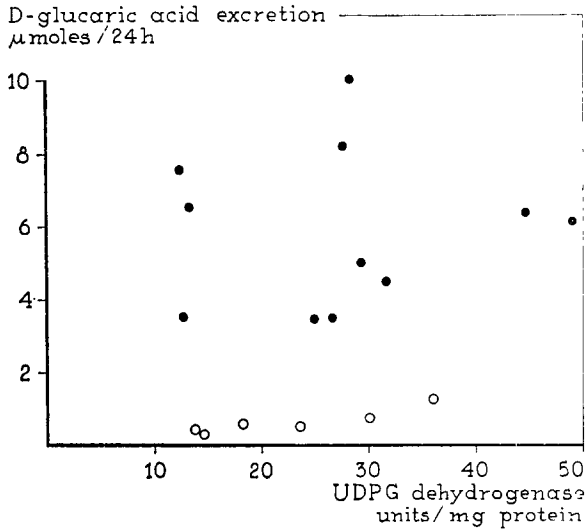

FIG. 2. Diagram representing urinary D-glucaric acid excretion and liver UDPG dehydrogenase activity for seventeen guinea pigs. Female guinea pigs weighing 300–400 g were divided into two groups. One group of eleven animals (●) received 150 mg/kg sodium barbital i.p., the other group of six animals (○) received saline. Urine was collected after 24 hr, the animals were sacrificed, and liver UDPG dehydrogenase activity and urinary D-glucaric acid excretion were determined. There was no correlation between the two quantities according to Kendall's rank correlation test (treated animals $P > 0{\cdot}95$, non-treated animals $P = 0{\cdot}10$).

TABLE 1. EFFECTS OF BARBITAL AND CELITE PRETREATMENT ON ENZYME ACTIVITIES* OF GUINEA PIG AND RAT LIVER HOMOGENATES

Pretreatment	Species	†Urinary excretion μmoles/24 hr		UDPG dehydrogenase (units)	D-glucuronolactone dehydrogenase 10^2 μmoles/hr	Demethylation activity 10^3 μmoles/hr	Tyrosine aminotransferase activity (units)
		L-ascorbic acid	D-glucaric acid				
Sodium barbital 150 mg/kg i.p. 24 hr before sacrifice	guinea pig,‡ female (11)		5·7 ±2·2	25·7 ± 11·1	4·9 ± 2·6	27·3 ± 4·5	10·4 ± 5·2
Control	(6)		0·60 ± 0·32	21·3 ± 8·1	3·4 ± 0·8	12·1 ± 2·3	9·2 ± 1·7
P value§			<0·01	>0·25	>0·10	<0·01	>0·25
Sodium barbital 150 mg/kg i.p. 24 hr before sacrifice	rat,‡ female (6)	32·3 ± 18·4	2·1 ± 0·6	18·2 ± 6·1	2·9 ± 0·5	18·4 ± 4·6	6·8 ± 1·1
Control	(6)	12·8 ± 8·4	0·42 ± 0·11	16·5 ± 2·4	2·3 ± 0·3	10·3 ± 2·1	7·8 ± 1·5
P value§¶		0·03	<0·01	0·25	0·04	<0·01	>0·10
Celite 60 mg/kg i.p. 24 hr before sacrifice	rat,‡ female (6)	10·7 ± 4·2	0·51 ± 0·14	18·0 ± 4·3	2·6 ± 0·5	9·8 ± 2·0	31·5 ± 9·7
P value§¶		>0·25	>0·25	>0·10	>0·25	>0·25	<0·01

* Activities per mg protein determined as described under Methods; means with S.D. are shown.

† Collection of urine was started immediately after injection.

‡ Guinea pigs weighing 300–400 g and rats weighing 180–200 g were used. Numbers in parentheses indicate number of animals on which each mean is based. Control animals were given saline i.p.

§ *P* values were obtained by applying the two-sided Wilcoxon two-sample test.

¶ Pretreatment with celite and sodium barbital was done simultaneously. Both groups were compared with the same control group.

could be the result of an enhanced activity of liver D-glucuronolactone dehydrogenase, the activity of this enzyme was also studied. Drugs stimulating the glucuronic acid pathway also induce enhanced microsomal drug metabolism.[1] Liver demethylation activity was determined in rats and guinea pigs in order to check whether barbital treatment was adequate for induction (Table 1). Excretion of D-glucaric acid and L-ascorbic acid as well as microsomal demethylation activity were strongly enhanced, D-glucuronolactone dehydrogenase activity was slightly increased, and UDPG dehydrogenase activity was not changed.

In order to determine whether stimulation of the glucuronic acid pathway occurred in a stress situation, rats were treated with celite. Activity of tyrosine aminotransferase[23] was determined in order to check whether a stress situation was achieved. Tyrosine aminotransferase was induced, but no effect on excretion of the acids or on activities of UDPG dehydrogenase, D-glucuronolactone and microsomal demethylation could be observed (Table 1).

In the preceding experiments enzymic assays were made 24 hr after injection of drug. It was shown by Steiner[24] that the induction of UDPG-glycogen glucosyltransferase (2.4.1.11) upon refeeding of fasting rats which reached maximal activity after 3 hr, had disappeared after 20 hr. A similar effect might occur for UDPG dehydrogenase

TABLE 2. UDPG DEHYDROGENASE ACTIVITY AND D-GLUCURONOLACTONE DEHYDROGENASE ACTIVITY OF GUINEA PIG LIVER HOMOGENATES AT DIFFERENT TIMES AFTER ADMINISTRATION OF BARBITAL

Time after administration* (hr)		UDPG dehydrogenase† units	*P* value	D-glucuronolactone dehydrogenase* (10^2 μmoles/hr)	*P* value
5	treated (6)	24·5 ± 3·8	0·05	2·7 ± 0·9	>0·25
	control (6)	28·5 ± 2·9		3·4 ± 0·9	
9	treated (6)	23·0 ± 5·3	0·09	4·2 ± 1·6	>0·10
	control (6)	15·7 ± 8.0		2·8 ± 1·6	
24	treated (11)	25·7 ± 11·1	>0·25	4·9 ± 2·6	>0·10
	control (6)	21·3 ± 8·1		3·4 ± 0·8	
72	treated (6)	23·8 ± 3·6	>0·25	2·7 ± 1·0	>0·25
	control (6)	23·5 ± 4·8		3·0 ± 0·7	

* Female guinea pigs weighing 300–400 g were given single injections of 150 mg/kg sodium barbital i.p. at zero time. The 72-hr group was given three injections: at zero time, at 24 hr and at 48 hr. The control animals were given saline. Number in parentheses indicate number of animals on which mean is based.

† Activities and *P* values are expressed as in Table 1.

after barbital treatment. Examination of UDPG dehydrogenase and D-glucuronolactone dehydrogenase activities at 5–72 hr after administration of barbital did not show any significant effects (Table 2). Chloretone treatment during 6 days enhanced UDPG dehydrogenase activity and L-ascorbic acid excretion as found by other investigators.[10, 11] Twenty hours after a single dose of chloretone an increase in L-ascorbic

acid excretion was noticed, but UDPG dehydrogenase activity did not differ significantly from the control value (Table 3). This suggests that the enhanced UDPG dehydrogenase activity is of minor importance for the stimulation.

In order to evaluate the role of *de novo* enzyme synthesis in the stimulation by barbital, the effects of inhibitors of protein synthesis were determined. Rats were

TABLE 3. EFFECT OF CHLORETONE PRETREATMENT ON UDPG DEHYDROGENASE ACTIVITY OF RAT LIVER HOMOGENATES AND ON L-ASCORBIC ACID EXCRETION IN RAT URINE

	Pretreatment*		UDPG dehydrogenase†	*P* value	L-ascorbic acid excretion‡ (μmoles/24 hr)	*P* value
I	150 mg/kg oral, daily for 6 days	(6)	30·8 ± 13·3	0·03	39·9 ± 21·6	<0·01
II	150 mg/kg oral, single dose	(8)	18·8 ± 6·5	>0·25	23·3 ± 8·6	<0·01
III	Control	(7)	16·1 ± 3·6		3·5 ± 2·5	

* Female rats, weighing 180–220 g were used. Group I was sacrificed 24 hr after the last dose, group II was sacrificed 20 hr after a single dose. Animals in group I and II were compared with control animals in group III.

† Activities and *P* values are expressed as in Table 1.

‡ Collection of urine was started immediately after last dose.

injected with puromycin and actinomycin D in quantities high enough to prevent protein synthesis, and urinary excretion of D-glucaric acid and L-ascorbic acid was determined. The data in Table 4 show that puromycin and actinomycin D did not prevent the effect of barbital, suggesting that *de novo* enzyme synthesis is not involved in it.

DISCUSSION

In previous reports little attention was paid to the time course of drug stimulation of the glucuronic acid pathway.[1, 3–5, 10] It appears from the present experiments that stimulation occurs within 3 hr after barbital administration to rats (Table 4).

Marsh[16] described a new aspect concerning the metabolism of D-glucuronic acid. He observed that in mammalian systems D-glucuronolactone or D-glucuronic acid is partly metabolized to D-glucaric acid, and identified the operative enzyme as D-glucuronolactone dehydrogenase (1.1.1.70). Our observation that after barbital treatment D-glucuronolactone dehydrogenase activity is not significantly enhanced in guinea pigs and only slightly enhanced in rats is in agreement with the concept that enhanced D-glucaric acid excretion results from an increased formation of D-glucuronic acid.[3]

In contrast to previous investigators,[7] we did not observe an increase in UDPG dehydrogenase activity after barbital treatment. The inability of puromycin and actinomycin D to block barbital-stimulated excretion of D-glucaric acid and L-ascorbic acid indicates that the mechanism of stimulation does not involve increased enzyme synthesis. The enhanced UDPG dehydrogenase activity after chronic chloretone treatment appears to be only of secondary importance.

TABLE 4. BARBITAL STIMULATED URINARY EXCRETION OF L-ASCORBIC ACID AND D-GLUCARIC ACID* DURING TREATMENT WITH PUROMYCIN AND ACTINOMYCIN D

Treatment†	3 hr Before experiment	First 3 hr during experiment	*P* value‡	Second 3 hr during experiment	*P* value‡
I Barbital with puromycin (6)	A 0·68 ± 0·38	1·67 ± 0·53	0·03	3·66 ± 0·95	0·03
	B 0·044 ± 0·009	0·143 ± 0·036	0·03	0·174 ± 0·022	0·03
II Barbital with actinomycin D (6)§	A 0·60 ± 0·41	1·02 ± 0·31	0·06	5·23 ± 2·64	0·03
	B 0·029 ± 0·006	0·049 ± 0·017	>0·10	0·165 ± 0·104	0·03
III Barbital alone (8)	A 0·80 ± 0·43	2·11 ± 1·43	0·03	3·75 ± 1·43	0·03
	B 0·035 ± 0·008	0·096 ± 0·045	0·03	0·222 ± 0·066	0·03

* A: L-ascorbic acid; B: D-glucaric acid excretion (μmoles per 3 hr).

† Male weanling rats, weighing 45–60 g, were divided into three groups. After collecting urine over a 3-hr period the rats were treated as follows:

Group I was given injections of 140 mg/kg of sodium barbital at zero time, together with injections of puromycin 30 mg/kg. The puromycin injections were repeated every hr.

Group II was given twice actinomycin D 800 μg/kg at zero time and at 3 hr. The group was given injections of 140 mg/kg sodium barbital at 30 min.

Group III was treated as group I except that puromycin was omitted. Urine was collected in two consectuvie 3-hr periods. Means with standard deviations are shown. Numbers in parentheses indicate number of animals in each group.

‡ *P* values were obtained by applying the two-sided Wilcoxon signed-rank test. The two 3-hr intervals after treatment were compared with the 3-hr interval before treatment. Comparison of the excretion of the acids of groups I and II with group III did not show significant differences in the second 3-hr interval and in the control 3-hr interval ($P > 0{\cdot}10$).

§ Treatment of a group of six rats with only actinomycin D did not influence significantly urinary excretion of the acids during the first 6 hr after injection.

Apart from *de novo* enzyme synthesis other explanations of the stimulation of the glucuronic acid pathway can be considered. It has been found that barbital inhibits the *in vitro* conversion of D-glucuronolactone to L-gulonolactone[25] and to D-glucaric acid,[26] and of UDP-glucuronic acid to D-glucuronic acid-1-phosphate.[7] The concentrations of barbital in these experiments were the same as those expected to occur *in vivo*. The enhanced excretion of L-ascorbic acid and D-glucaric acid during barbital treatment in the face of the *in vitro* inhibition of the conversions just mentioned suggests that the activities of the concerning enzymes are not rate-limiting. It is unlikely that this kind of non-specific inhibitions in other pathways would give an increased supply of UDPG or UDP-glucuronic acid. The exact sequence of steps leading from UDP-glucuronic acid to D-glucuronic acid have not been definitively elucidated. A better knowledge of the role of the liver phosphatase converting D-glucuronic acid-1-phosphate to D-glucuronic acid is needed.[27, 28] There is no definitive information whether UDP-glucuronic acid may also yield D-glucuronic acid through the successive actions of UDP glucuronyltransferase and β-glucuronidase.[29] A fuller understanding of the mechanism of stimulation by barbiturate will require more knowledge on these points.

The stress condition after treatment with celite is associated with an increased concentration of hydrocortisone in the liver.[23] The enhanced activity of tyrosine aminotransferase in the liver is attributable to hormonal induction by hydrocortisone. Stimulation of the glucuronic acid pathway and enhanced microsomal demethylation activity were not observed in rats under celite stress. Hence there is no indication that hydrocortisone release resulting from stress has any effect on these systems. While we could confirm the induction of tyrosine aminotransferase by celite, observed by Kenney and Flora,[23] we could not detect an enhanced activity of tyrosine aminotransferase after barbital treatment. This suggests that barbital does not act via a stress response of the adrenals. This is in agreement with the observation of Burns *et al.*[30] that barbital can stimulate L-ascorbic acid excretion in adrenalectomized rats.

It has been shown that therapeutic doses of phenobarbital in man induced enhanced drug metabolism[31] as well as increased D-glucaric acid excretion.[4] Both aspects of enhanced metabolism evoked by phenobarbital also occurred in rats.[32] Other drugs, capable of inducing drug metabolism also stimulate the glucuronic acid pathway in rats as indicated by increased L-ascorbic acid formation. This suggested a possible relationship between the effects on drug metabolism and glucuronic acid pathway. Conney and Gilman,[33] and Ernster and Orrenius[34] have shown conclusive evidence for the assumption that the induction of drug metabolizing enzyme systems by phenobarbital is caused by *de novo* enzyme synthesis. It is reasonable to expect that the inability of puromycin and actinomycin D to block the increase of D-glucaric acid and L-ascorbic acid by barbital would also occur with phenobarbital. We may, therefore, conclude that the stimulation of the glucuronic acid pathway by barbiturate occurs by a mechanism entirely different from the induction of drug metabolizing enzyme systems.

Acknowledgements—We are grateful to Merck, Sharp and Dohme Inc., New York, for a gift of actinomycin D. The technical assistance of Miss M. van Mierlo and Miss A. Aarnoutse is acknowledged.

REFERENCES

1. J. J. Burns, A. H. Conney, P. G. Dayton, C. Evans, G. R. Martin and D. Taller, *J. Pharmac. exp. Ther.* **129**, 132 (1960).
2. M. Enklewitz and M. Lasker, *J. biol. Chem.* **110**, 443 (1935).
3. C. A. Marsh and L. M. Reid, *Biochim. biophys. Acta* **78**, 726 (1963).
4. E. M. Aarts, *Biochem. Pharmac.* **14**, 359 (1965).
5. C. Evans, A. H. Conney, N. Trousof and J. J. Burns, *Biochim. biophys. Acta* **41**, 9 (1960).
6. O. Touster, R. W. Hester and R. A. Siler, *Biochem. biophys. Res. Commun.* **3**, 248 (1960).
7. S. Hollmann and O. Touster, *Biochim. biophys. Acta* **62**, 338 (1962).
8. S. Villa-Trevino, K. H. Schull and E. Farber, *J. biol. Chem.* **238**, 1757 (1963).
9. H. Bartels and H. J. Hohorst, *Biochim. biophys. Acta* **71**, 214 (1963).
10. A. H. Conney, G. A. Bray, C. Evans and J. J. Burns, *Ann. N.Y. Acad. Sci.* **92**, 115 (1961).
11. I. M. Arias, L. Gartner, M. Furman and S. Wolfson, *Proc. Soc. exp. Biol. Med.* **112**, 1037 (1963).
12. O. Touster, S. Hollmann, O. Pineda and S. Shumaker, *Proc. 1st Int. Pharmacol. Meeting* **VI**, 47. Pergamon Press, New York (1962).
13. P. G. Dayton, P. Vrindten and J. M. Perel, *Biochem. Pharmac.* **13**, 143 (1964).
14. F. de Matteis, *Biochim. biophys. Acta* **82**, 641 (1964).
15. O. C. De Barreiro, *Biochem. Pharmac.* **14**, 1694 (1965).
16. C. A. Marsh, *Biochem. J.* **87**, 82 (1963).
17. C. A. Marsh, *Biochem. J.* **86**, 77 (1963).
18. J. Gorsky, Y. Aizawa and G. C. Mueller, *Archs. Biochem. Biophys.* **95**, 508 (1961).
19. J. L. Strominger, E. S. Maxwell, J. Axelrod and H. M. Kalckar, *J. biol. Chem.* **224**, 79 (1957).
20. J. A. Smith, W. J. Waddell and F. C. Butler, *Life Sci.* **7**, 486 (1963).
21. F. T. Kenney, *J. biol. Chem.* **234**, 2707 (1959).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
23. F. T. Kenney and R. M. Flora, *J. biol. Chem.* **236**, 2699 (1961).
24. D. F. Steiner, *Nature, Lond.* **204**, 1171 (1964).
25. Y. Mano, K. Suzuki and N. Shimazono, *J. Biochem., Tokyo* **49**, 618 (1961).
26. C. A. Marsh, *Biochem. J.* **89**, 108 (1963).
27. A. H. Conney and J. J. Burns, *Biochem. biophys. Acta* **54**, 369 (1961).
28. S. Takanashi, K. Ihda and M. Kawada, *J. Biochem., Tokyo* **59**, 78 (1966).
29. B. M. Pogell and L. F. Leloir, *J. biol. Chem.* **236**, 293 (1961).
30. J. J. Burns, C. Evans and N. Trousof, *J. biol. Chem.* **227**, 785 (1957).
31. S. A. Cuccinell, A. H. Conney, M. Sansur and J. J. Burns, *Clin. Pharmac. Ther.* **6**, 420 (1965).
32. A. H. Conney and J. J. Burns, *Nature, Lond.* **184**, 363 (1959).
33. A. H. Conney and A. G. Gilman, *J. biol. Chem.* **238**, 3682 (1963).
34. L. Ernster and S. Orrenius, *Fedn Proc.* **24**, 1190 (1965).