Biochemical Pharmacology, 1966, Vol. 15, pp. 573–581. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF *o,p'*DDD* ON CORTISOL AND HEXOBARBITAL METABOLISM

D. KUPFER and L. PEETS

Department of Chemical Pharmacology,
Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y., U.S.A.

(*Received* 7 *September* 1965; *accepted* 5 *November* 1965)

Abstract—The administration of 2,2-(*o*-chlorophenyl,*p*-chlorophenyl)-1,1-dichloroethane (*o,p'*DDD) to both immature and adult male rats resulted in the stimulation of both cortisol and hexobarbital metabolism by 9000 *g* supernatant of liver homogenate supplemented with a NADPH-generating system.

The possible relation of this finding to the stimulation *in vivo* by *o,p'*DDD of cortisol metabolism in the guinea pig and in the human is discussed.

RECENTLY we reported that in the guinea pig 2,2-(*o*-chlorophenyl,*p*-chlorophenyl)-1,1-dichloroethane (*o,p'*DDD) caused a stimulation of cortisol metabolism, yielding an increase in urinary polar metabolites.[1] Bledsoe *et al.*[2] observed a similar action of *o,p'*DDD on cortisol metabolism in human patients; there was a substantial increase in the urinary excretion of 6β-hydroxycortisol without a significant decrease in cortisol production rate.

The findings by Hart and Fouts[3] and by Ghazal *et al.*[4] that the administration of DDT to rats stimulated the metabolism of hexobarbital by microsomal liver enzymes and our observations that DDT stimulated cortisol metabolism[5] in the guinea pig suggested a similar mechanism of action for *o,p'*DDD.

Our attempts to study the effect of *o,p'*DDD on the microsomal hexobarbital metabolism of guinea pig were not successful; in fact, we failed to obtain an active system that would metabolize hexobarbital. These studies were therefore carried out in the rat. The present work describes the correlation between the increase in rates of both hexobarbital and cortisol metabolism by 9000 *g* supernatant of liver homogenate from male rats treated with *o,p'*DDD. The possible significance of these findings is discussed.

While this work was in progress, Straw *et al.*[6] reported that *o,p'*DDD treatment stimulated pentobarbital metabolism in the rat.

EXPERIMENTAL

Materials and methods

Animals. Male albino rats of the Wistar strain obtained at Royal Hart Farms were fed *ad libitum* with Purina chow and water. Immature animals (c. 60 g body weight) and adult animals (c. 200 g body weight) were dosed orally with 300 mg *o,p'*DDD/kg for three and nine days respectively. Treated animals received *o,p'*DDD in a suspension of 0·5% carboxymethylcellulose solution containing about 2% polysorbate

* 2,2-(*o*-Chlorophenyl,*p*-chlorophenyl)-1,1-dichloroethane.

80; controls received the vehicle alone. The animals were decapitated 24 hr after the last dose. The livers were immediately removed, rinsed, blotted, and homogenized in 3 volumes of 1·15% KCl in a Potter-Elvehjem glass homogenizer. The liver homogenate was centrifuged at 9000 *g* in a refrigerated Servall centrifuge for 20 min. Supernatant was decanted and stored at −20° until used. Microsomes were prepared by the method of McGuire and Tomkins.[7]

Hexobarbital metabolism. The incubations were conducted at 37° for 30 min with a mixture containing 0·5 or 1·0 ml liver supernatant, hexobarbital (1·93 μmoles), NADP (2 μmoles), glucose-6-phosphate (40 μmoles), nicotinamide (50 μmoles), $MgCl_2$ (75 μmoles), and phosphate buffer, pH 7·4 (250 μmoles), in a total volume of 5 ml. The assay of residual unmetabolized hexobarbital was carried out as described by Cooper and Brodie.[8]

Cortisol metabolism. 4-^{14}C-Cortisol (254 or 504 μg; 0·5 μc) in methanolic solution containing 2% propylene glycol was placed in 25-ml Erlenmeyer flasks and evaporated to dryness under N_2. The Erlenmeyer flasks were chilled in an ice bath and the following substituents were added: 1 ml of 9000 *g* liver supernatant or liver microsomes* (0·33 g liver equivalent), NADP (2 μmoles), glucose-6-phosphate (40 μmoles), nicotinamide (50 μmoles), $MgCl_2$ (75 μmoles), and phosphate buffer, pH 7·4 (200 μmoles), in a final volume of 5 ml.

Zero-time samples were extracted immediately while the rest were incubated with shaking in air at 37° for 20 min and then extracted.

Extraction procedure. To the 5-ml incubation mixture was added 1 g NaCl, and the resulting suspension was extracted twice with 3 volumes of cold ethylacetate. The ethylacetate extract was washed twice with cold 0·1 N NaOH followed by immediate washing with small amounts of cold deionized water until neutral to litmus. The ethylacetate phase was dried over sodium sulfate and evaporated to dryness under reduced pressure. The residue was suspended in 1 ml MeOH; 0·05 ml of the methanolic solution, supplemented with 100 μg non-radioactive cortisol, was streaked across a 1-inch width Whatman 1 paper and developed by descending chromatography with a solvent system described by Burstein and Kimball[9] until the solvent front reached the end of the paper (4–6 hr). After chromatography the paper strips were dried in air, and the cortisol area was visualized under short-wave u.v. light. The chromatograms were cut into 2-cm segments starting 2·5 cm above the origin; each segment was then cut into 12 parts, placed in a vial, 5 ml of phosphor† was added, and radioactivity was determined with the Packard Tri-Carb scintillation spectrometer. The extent of metabolism (μmoles/g liver) was calculated from the per cent of counts in the different radioactive zones.

RESULTS

Figure 1 demonstrates the pattern of metabolism of 4-^{14}C-cortisol by 9000 *g* supernatant of immature rat liver. Two radioactive zones, A and B, in addition to a zone containing residual unreacted cortisol, were observed. The formation of these

* Glucose-6-phosphate dehydrogenase (280 Buchner units, obtained from Calbiochem, Los Angeles, Calif.) was present and nicotinamide was absent when incubations were carried out with microsomes.

† Four g of 2,5-diphenyloxazole and 0·050 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene dissolved in 1 liter toluene.

metabolites was found to be NADPH dependent. An increase in the formation of the most polar zone, A, was observed after *o,p'*DDD treatment.

Chromatography of extracts from incubation of cortisol with 9000 *g* supernatant from adult rat livers indicated a metabolic pattern similar to that of the immature;

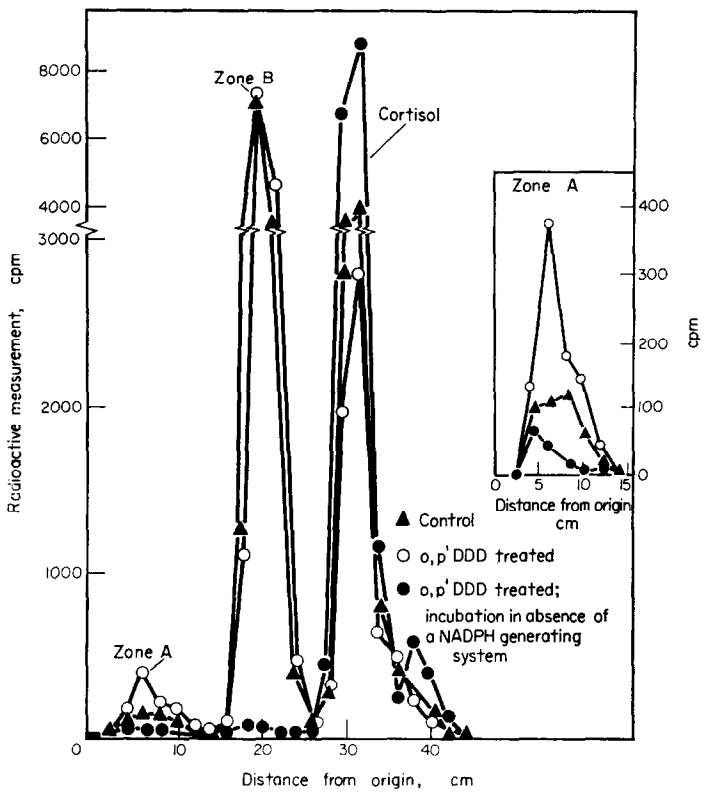

FIG. 1. Chromatographic profile of cortisol metabolites from incubations of labeled cortisol with 9000 *g* liver supernatant from immature male rats.

however, there was a quantitative difference (Fig. 2). There was an increase in the formation of zone A with a concomitant decrease in zone B in the adult animal. Treatment with *o,p'*DDD, in the adult rat, resulted in an increase in cortisol metabolism, as observed by the decrease in unmetabolized cortisol. A similar incubation with liver microsomes indicated a lower rate of cortisol metabolism as compared with that obtained with 9000 *g* supernatant; the chromatographic pattern of the radioactive metabolites was identical with that obtained with liver supernatant incubations, indicating that microsomes can carry out these reactions.

Zone A from cortisol incubations with adult liver 9000 *g* supernatant or microsomes yielded a single compact radioactive area even after 40-hr chromatography* (Fig. 3). The major portion of this area was more polar than 6β-hydroxycortisol (6β-OH-F); however, a small shoulder (amounting to about 5% of the radioactivity in zone A) with the chromatographic mobility of 6β-OH-F was also observed.

* Within this period, cortisol and zone B were eluted off the paper chromatograms.

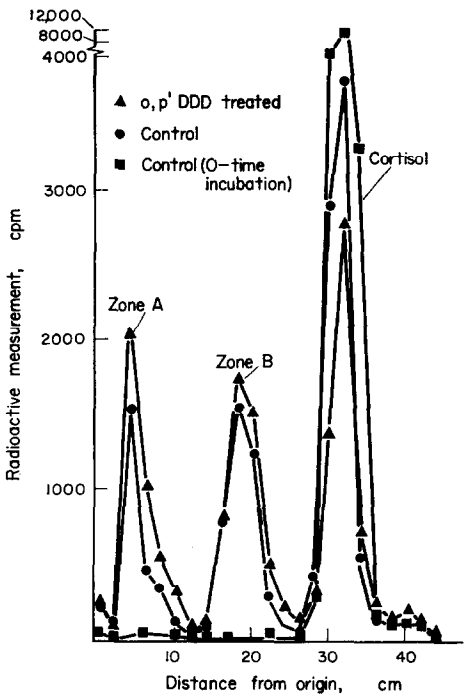

FIG. 2. Chromatographic profile of cortisol metabolites from incubations of labeled cortisol with 9000 *g* liver supernatant from adult male rats.

TABLE 1. COMPARISON OF LIVER MICROSOMAL ACTIVITY IN THE IMMATURE AND ADULT MALE RAT

Animals*		Hexobarbital† metabolism (μmoles/g liver/30 min)	Cortisol metabolism† (μmoles/g liver/20 min)		
			Zone A formed	Zone B formed	Substrate metabolized
Immature					
	Controls(4)‡	0·75 ± 0·13	0·050 ± 0·004	1·15 ± 0·18	1·4 ± 0·07
	Treated(4)‡	3·54 ± 0·25	0·096 ± 0·004	1·15 ± 0·14	1·4 ± 0·1
Adult					
Exp. 1	Controls(2)	4·49 ± 0·07	0·33 ± 0·0	0·63 ± 0·04	1·09 ± 0·01
	Treated(2)	5·88 ± 0·03	0·51 ± 0·11	0·69 ± 0·03	1·36 ± 0·22
Exp. 2	Controls(2)	2·23 ± 0·24	0·27 ± 0·05	0·66 ± 0·02	1·07 ± 0·10
	Treated(2)	3·43 ± 0·05	0·51 ± 0·05	0·75 ± 0·09	1·36 ± 0·11
Exp. 3	Controls(3)	3·03 ± 0·23	0·30 ± 0·03	0·61 ± 0·04	1·03 ± 0·02
	Treated(3)	4·60 ± 0·20	0·50 ± 0·03	0·70 ± 0·05	1·38 ± 0·06

* Immature animals weighed c. 60 g; treated with 300 mg *o,p'*DDD/kg for 3 days. Adult animals weighed c. 200 g; treated with 300 mg *o,p'*DDD/kg for 9 days; controls received vehicle alone. Numbers in parentheses refer to number of animals. Values represent the calculated mean ± standard error.

† Hexobarbital, 5·8 μmoles/g liver, was used in incubations except for Exp. 1 where 11·6 μmoles were used. Cortisol metabolism was carried out at 37° with 4·20 μmoles and 2·13 μmoles/g liver in the young and adult rats respectively.

‡ Three animals were used for cortisol metabolism.

Table 1 shows that liver supernatant from the immature male rat metabolized both cortisol and hexobarbital at a low rate. Treatment with *o,p'*DDD resulted in a five fold increase in hexobarbital metabolism. However, less than a two fold increase in the formation of zone A was observed. Cortisol metabolism in the treated animals did not increase, as seen by the unaltered quantities of residual cortisol. An increase in liver to body weight ratio was observed in all treated animals.

The adult rats metabolized hexobarbital at a rate severalfold higher than in the immature animals; the degree of stimulation by *o,p'*DDD was not so pronounced in the adult as in the immature animal, suggesting that maximal stimulation had been reached. Similarly, the formation of cortisol metabolite(s) (zone A) in the controlled adult was about sixfold that in the immature rat; less than a twofold increase in the formation of zone A was observed in treated animals. There was little or no increase in the formation of zone B. The rate of cortisol metabolism decreased somewhat in the adult as compared to the immature rat; furthermore, the rate of formation of zone B decreased substantially, being about half that in the immature animal. While there was no detectable increase in cortisol metabolism in the treated immature, there was an increase of about 30% (significant at $P \leq 0{\cdot}01$) in the metabolism of cortisol in the treated adults.

Table 2 indicates an increase in both hexobarbital and NADPH oxidations after *o,p'*DDD treatment. There was a parallel increase in cortisol metabolism with both

TABLE 2. MICROSOMAL REACTIONS IN *o,p'*DDD-TREATED ADULT MALE RATS*

Animal No.†		NADPH oxidation (Δ OD_{340}/g liver/min)	Hexobarbital metabolism (μmoles/g liver/30 min)	Cortisol metabolism (μmoles/g liver/20 min) Zone A formed	Zone B formed	Substrate metabolized	Liver as % body wt.
Treated							
	1	0·059	5·85	0·71 (0·113)	0·72 (0·64)	1·56 (0·94)	5·2
	2	0·069	5·91	0·31 (0·059)	0·67 (0·39)	1·10 (0·56)	6·2
Control							
	3	0·033	4·42	0·33 (0·065)	0·65 (0·53)	1·07 (0·68)	4·5
	4	0·040	4·56	0·33 (0·082)	0·58 (0·58)	1·04 (0·74)	4·8

* Incubations were carried out with 11·6 μmoles hexobarbital or with 2·1 μmoles cortisol/g liver (supernatant); values in parentheses are from incubations of cortisol with microsomes. NADPH oxidation was examined with 2 mg NADPH/g liver (microsomes).

† Male rats weighing C. 180 g at the beginning of experiment and c. 230 g at end of experiment; they were treated with 300 mg *o,p'*DDD/kg for 9 days while controls received vehicle alone

9000 *g* supernatant and microsomes in animal No. 1; however, there was no such correlation in the other treated animal (No. 2). While liver preparations from animal No. 2 oxidized hexobarbital and NADPH at a similar rate to that of the other treated animal (No. 1), the rate of cortisol metabolism with either 9000 *g* supernatant or microsomes was similar to that obtained with controls (No. 3 and No. 4). An increase in liver to body weight ratio was observed in both treated animals.

Table 3 demonstrates that the formation of zone A by 9000 *g* supernatant from liver of a mature male rat was inhibited by SKF 525-A (β-diethylaminoethyl diphenylpropylacetate) at concentrations of 2×10^{-5} and 1×10^{-4} M, the inhibition being

69% and 75% respectively. There was no alteration in rate of cortisol metabolism; however, a concomitant increase in zone B was observed. Similarly the conversion of zone B* into polar metabolites (chromatographic mobility similar to zone A) by 9000 *g* supernatant from liver of a mature male rat was inhibited (45% inhibition) by 5×10^{-4} M SKF 525-A.

TABLE 3. THE EFFECT OF SKF 525-A ON CORTISOL METABOLISM BY LIVER SUPERNATANT FROM AN ADULT MALE RAT

SKF 525-A (M)	Cortisol metabolism* (μmoles/g liver/20 min) Zone A formed	Zone B formed	Substrate metabolized
	0·56	0·64	1·33
2×10^{-5}	0·18	0·99	1·27
1×10^{-4}	0·14	1·03	1·25

* Conditions were the same as in Table 1. Zero-time controls yielded only unmetabolized cortisol.

DISCUSSION

The present results indicate that administration of *o,p'*DDD to immature and adult male rats yielded an increase in both hexobarbital oxidation and cortisol metabolism. The degree of stimulation in the rate of hexobarbital metabolism was larger in the immature animals which normally metabolize hexobarbital at a much lower rate (Table 1). The formation of polar cortisol metabolites, zones A and B, was observed in all treated and controlled immature and adult animals. However, only the rate of formation of zone A was stimulated after treatment with *o,p'*DDD. This metabolite(s) was formed in almost negligible quantities in the controlled immature rat, while in the controlled adult it became a major metabolite (amounting to about 30% of total).

Zone A from rat liver incubations did not contain significant quantities of 6β-hydroxycortisol; in fact chromatography yielded only a single zone whose major component (95%) was more polar than 6β-hydroxycortisol (Fig. 3). This is contrary to observations obtained with guinea pig microsomal incubations in which the formation of 6β-hydroxycortisol as a major polar metabolite has been demonstrated.[10]

There was less than a twofold increase in the formation of zone A after treatment with *o,p'*DDD in both immature and adult rats; however, in terms of quantities formed, *o,p'*DDD treatment yielded in the immature animal almost negligible quantities of zone A, while in the adult this zone amounted to about 40% of total cortisol metabolites.

The rate of formation of zone B did not seem to increase significantly by *o,p'*DDD treatment in either the immature or the adult rat (Table 1). It is interesting to note that while the rate of metabolism of cortisol decreased slightly in the adult as compared with the immature, the rate of formation of zone B diminished to about half as compared with that of the immature animal.

* The radioactive area corresponding to zone B was localized on a chromatogram from an incubation of 4-^{14}C-cortisol with liver supernatant (see Experimental), eluted with methanol; representative aliquots used.

A limited attempt to characterize metabolites of zones A and B was made. Both zones were presumably composed of ring A reduced metabolites, as is evident from the lack of absorption of short-wave u.v. light on paper and the lack of absorption at 240 mμ of eluted substances in a methanolic solution. Both zones reduced blue tetrazolium, suggesting an intact α ketolic side chain. The chromatographic mobility of zone B was similar to that of 3α,11β,17α,21-tetrahydroxy-pregnan-20-one, while that of zone A was slower than that of 6β-hydroxycortisol.

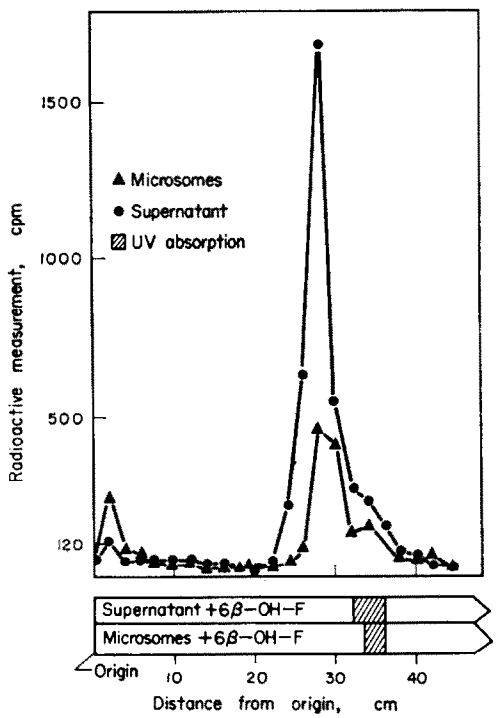

FIG. 3. Chromatograms of cortisol metabolites from incubations of labeled cortisol with liver supernatant or microsomes from adult male rat; developed for 40 hr by descending chromatography.

The finding that SKF 525-A inhibits *in vitro* the rate of formation of zone A with a concomitant increase of zone B (Table 3) and inhibits the conversion of zone B into polar metabolites (presumably zone A) is of interest. SKF 525-A has been shown to inhibit steroid hydroxylase[11] but to have no effect on Δ^4-3 keto reduction* The above observations on structure, chromatographic mobilities, and SKF 525-A inhibition, together with the finding that treatment with *o,p'*DDD stimulated the formation of zone A but not of zone B, might be taken to suggest that zone A is a hydroxylated ring A reduced cortisol metabolite, while zone B is a ring A reduced product; however, further work is required to demonstrate the correctness of these speculations.

Recently Conney and Klutch[12] and Kuntzman *et al.*[11] demonstrated the similarity between oxidative drug-metabolizing enzymes and certain steroid hydroxylases.

* D. Kupfer and L. Peets (unpublished results).

These authors suggested that these enzyme systems might be identical. In fact, Tephly and Mannering[13] showed that steroids such as estradiol, testosterone, and cortisol competitively inhibited the metabolism of ethylmorphine. The work, however, did not demonstrate whether steroids are alternative substrates or competitive inhibitors. Our finding with a single *o,p'*DDD-treated animal (and therefore entirely inconclusive) that the rate of hexobarbital and NADPH oxidations did not correlate well with the rate of cortisol metabolism (by either liver 9000 *g* supernatant or microsomes) when compared to that of controlled and other *o,p'*DDD-treated animals, suggests that these enzymes might be different. Such a single finding would be normally overlooked since metabolism studies are often carried out with a mixture of pooled livers from several animals. A comparison of these enzymes in the immature and in the adult animal provides further evidence for certain dissimilarities in these enzyme systems. Thus, while the rate of hexobarbital metabolism in the *o,p'*DDD-treated immature animals was similar to rates obtained in controlled adult animals, the rate of formation of the polar cortisol metabolite, A, remained minimal in both controlled and treated immature animals and reached significant proportions only in the adult animal.

The increase in liver weight after *o,p'*DDD treatment suggests that the stimulation of microsomal enzymes might occur via a mechanism similar to that reported for other stimulators of microsomal enzymes; namely, by an increase in the *de-novo* synthesis of these enzymes.[14, 15] The study by Straw *et al.*[6] with ethionine inhibition of *o,p'*DDD stimulation of pentobarbital metabolism suggested an increase in enzyme synthesis. Unfortunately, ethionine in that study exhibited certain hepatotoxic activity which would render these observations inconclusive.

The present work indicates that the activity of *o,p'*DDD resembles, in several aspects, the activity of certain other compounds such as phenobarbital and diphenylhydantoin. The similarity between the action of these compounds rests in their similar stimulatory activity on microsomal "drug-metabolizing" reactions and cortisol transformations both *in vivo* and *in vitro*.

Thus, *,o'p*DDD stimulated, *in vivo*, the transformation of cortisol to polar metabolites in the guinea pig[1] and in man[2] and increased the rate of hexobarbital and cortisol metabolism by rat liver preparations *in vitro* (this report). Similarly, phenobarbital, known to stimulate microsomal enzymatic activity in the rat, was recently shown to stimulate 6β-hydroxylation of cortisol *in vivo* in the human[16] and *in vitro* in the guinea pig.[10] The administration of diphenylhydantoin caused an increase in urinary 6β-hydroxycortisol in man[17] and the stimulation of the rate of 6β-hydroxylation of cortisol by guinea pig liver microsomes;[10] however, this compound, in contrast to *,o'p*DDD and phenobarbital, did not stimulate in the rat *in vitro* the cortisol metabolism.[18]

The observation that DDT stimulates the transformation of cortisol to urinary polar metabolites in the guinea pig[5] and hexobarbital metabolism both *in vivo* and *in vitro* in the rat[3, 4] suggests the possibility that other chlorinated insecticides which stimulate microsomal enzymes might possess similar activity toward cortisol metabolism. Whether DDT acts directly or via one of its metabolites is not known.

The present findings that *,o'p*DDD stimulates *in vitro* the metabolism of cortisol in the male rat and the observations that this drug stimulates *in vivo* cortisol metabolism in the guinea pig[1] and in the human,[2] suggest that the increase *in vivo* in

cortisol metabolism results from an increase in the liver microsomal enzymes.

Acknowledgements—The authors would like to thank Dr. D. A. Buyske for stimulating discussi on and Mrs. Doris Lennon for the help extended in preparing the manuscript.

REFERENCES

1. D. KUPFER, T. BALAZS and D. A. BUYSKE, *Life Sci.* **3**, 959 (1964).
2. T. BLEDSOE, D. P. ISLAND, R. L. NEY and G. W. LIDDLE, *J. clin. Endocr.* **24**, 1303 (1964).
3. L. G. HART and J. R. FOUTS, *Proc. Soc. exp. Biol. (N.Y.)* **114**, 388 (1963).
4. A. GHAZAL, W. KORANSKY, J. PORTIG, H. W. VOHLAND and I. KLEMPAU, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **249**, 1 (1964).
5. T. BALAZS and D. KUPFER, *Toxicol. appl. Pharmacol.* In press.
6. J. A. STRAW, I. W. WATERS and M. J. FREGLY, *Proc. Soc. exp. Biol. (N.Y.)* **118**, 391 (1965).
7. J. S. MCGUIRE and G. M. TOMKINS, *J. biol. Chem.* **235**, 1634 (1960).
8. J. R. COOPER and B. B. BRODIE, *J. Pharmacol. exp. Ther.* **114**, 409 (1955).
9. S. BURSTEIN and H. L. KIMBALL, *Analyt. Biochem.* **4**, 132 (1962).
10. A. H. CONNEY, M. JACOBSON, K. SCHNEIDMAN and R. KUNTZMAN, *Life Sci.* **4**, 1091 (1965).
11. R. KUNTZMAN, M. JACOBSON, K. SCHNEIDMAN and A. H. CONNEY, *J. Pharmacol. exp. Ther.* **146**, 280 (1964).
12. A. H. CONNEY and A. KLUTCH, *J. biol. Chem.* **238**, 1611 (1963).
13. T. R. TEPHLY and G. J. MANNERING, *Pharmacologist* **6**(2), 186 (1964).
14. A. H. CONNEY, E. C. MILLER and J. A. MILLER, *Cancer Res.* **16**, 450 (1956).
15. H. V. GELBOIN and N. R. BLACKBURN, *Cancer Res.* **24**, 356 (1964).
16. S. BURSTEIN and W. L. KLAIBER, *J. clin. Endocr.* **25**, 293 (1965)
17. E. E. WERK, JR., J. MACGEE and L. J. SHOLITON, *J. clin. Invest.* **43**, 1824 (1964).
18. L. J. SHOLITON, E. E. WERK, JR. and J. MACGEE, *Metabolism* **13**, 1382 (1964).