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EFFECTS OF STEROID HORMONES *IN VITRO* ON ADRENAL XENOBIOTIC METABOLISM IN THE GUINEA PIG

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Abstract—Studies were carried out to determine the effects of steroid hormones *in vitro* on adrenal and hepatic microsomal benzphetamine demethylation and benzo[*a*]pyrene hydroxylation. Testosterone inhibited adrenal drug metabolism but had no effect on hepatic enzymes, whereas 6β-hydroxytestosterone had no effect in either tissue. All of the corticosteroids tested (cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, progesterone, and 17-hydroxyprogesterone) produced a concentration-dependent inhibition of adrenal drug metabolism, but had little or no effect on hepatic metabolism. The 17-deoxy-steroids were more potent inhibitors of adrenal metabolism than were their 17-hydroxylated counterparts. Cortisol was a potent inhibitor of adrenal benzphetamine and benzo[*a*]pyrene metabolism, produced a type I difference spectrum in adrenal microsomes, and diminished the magnitude of the benzphetamine-induced spectrum; 6β-hydroxycortisol had none of these effects. Prior addition of benzphetamine to adrenal microsomes reduced the size of cortisol-induced spectral change. The results demonstrate that the effects of corticosteroids *in vitro* are relatively specific for adrenal enzymes and established a close association between the 6β-hydroxylase and some drug-metabolizing enzymes. Adrenal steroids may have an important role in the regulation of adrenal xenobiotic metabolism.

For many years adrenal cytochrome P-450-containing enzymes (mixed function oxidases) were thought to be highly specific for endogenous steroid substrates and to function only in steroid hormone biosynthesis (see refs. 1 and 2). However, it is now well known that many drugs, carcinogens and other xenobiotics are also metabolized by adrenal microsomes [3–17]. In fact, some foreign substances are metabolized far more rapidly by adrenal than by hepatic microsomes. Adrenal drug-metabolizing activity has been found to be particularly high in the human and monkey fetus [5–10] and in both fetal and adult guinea pigs [11–16].

Although adrenal xenobiotic metabolism has been investigated in a number of laboratories, there is still relatively little known about its overall significance and regulation by physiological and pharmacological factors. Among the questions to be resolved is the relationship between drug-metabolizing and steroid-hydroxylating mixed function oxidases in the adrenal cortex. It is not known, for example, if different cytochrome P-450 moieties are responsible for the metabolism of exogenous and endogenous substrates by adrenal microsomes. Multiple species of cytochrome P-450 with varying substrate specificities have been identified in hepatic microsomes [18, 19] and in adrenal mitochrondria [20, 21] but not in adrenal microsomes. If adrenal microsomal mixed function oxidases have overlapping substrate specificities, steroids may play an important role in the regulation of adrenal xenobiotic metabolism by serving as competitive substrates. Such effects would be of particular significance in an organ like the adrenal cortex which normally contains high concentrations of steroids. The following studies were, therefore, carried out to evaluate the direct effects of steroids on adrenal xenobiotic metabolism *in vitro*. In addition, by comparing the actions of structurally related steroids, we have attempted to determine which of the steroid hydroxylases are most closely associated with adrenal drug-metabolizing enzymes. Effects on hepatic metabolism were also examined to determine the specificity of steroid actions on adrenal enzymes.

METHODS

Adult (750–900 g), male English Short Hair guinea pigs were obtained from the Camm Research Institute (Wayne, NJ) and maintained under standardized conditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°) on a diet of Wayne Guinea Pig Diet and water *ad lib*. All guinea pigs were allowed at least 7 days to become acclimated to the housing conditions prior to being killed.

Animals were decapitated between 8:00 and 9:00 a.m. Adrenal glands and livers were quickly removed and microsomes were prepared as described previously [15]. Benzo[*a*]pyrene hydroxylation was determined by the fluorometric assay of Nebert and Gelboin [22]. Quinine sulfate was calibrated against authentic 3-hydroxybenzo[*a*]pyrene and routinely used as the fluorescence standard. The demethylation of benzphetamine by adrenal and hepatic microsomes was assayed as the production of formaldehyde [23], as described previously [15]. None of the steroids studied interfered with either the benzo[*a*]pyrene hydroxylase or benzphetamine demethylase assays.

Substrate-induced difference spectra in adrenal and hepatic microsomes were obtained using an

Table 1. Effects of testosterone, 6β-hydroxytestosterone, and estradiol on adrenal and hepatic benzphetamine demethylase and benzo[a]pyrene hydroxylase activities

Steroid conc (μM)	Benzphetamine demethylation*		Benzo[a]pyrene hydroxylation*	
	Adrenal	Liver	Adrenal	Liver
Control	100†	100‡	100§	100‖
Testosterone				
2	97 ± 4	99 ± 1	93 ± 4	99 ± 6
5	91 ± 4	99 ± 3	79 ± 6¶	97 ± 8
10	83 ± 5¶	97 ± 6	69 ± 4¶	108 ± 11
40	57 ± 5¶	91 ± 5	49 ± 3¶	103 ± 6
6β-OH-testosterone				
2	99 ± 4	100 ± 6	96 ± 6	102 ± 6
5	101 ± 5	97 ± 5	99 ± 5	98 ± 5
10	97 ± 6	102 ± 5	101 ± 5	96 ± 6
40	98 ± 5	97 ± 5	97 ± 7	101 ± 5
Estradiol				
2	97 ± 1	98 ± 1	97 ± 3	117 ± 4¶
5	95 ± 2	97 ± 2	111 ± 3¶	113 ± 7
10	95 ± 1¶	99 ± 2	120 ± 1¶	100 ± 7
40	86 ± 2¶	91 ± 2¶	122 ± 3¶	102 ± 2

* Values are expressed as mean per cent of control ± S.E.; six to eight determinations per value.
† Equivalent to 12.1 nmoles/min/mg protein.
‡ Equivalent to 2.2 nmoles/min/mg protein.
§ Equivalent to 625 pmoles/min/mg protein.
‖ Equivalent to 195 pmoles/min/mg protein.
¶ $P < 0.05$ (vs control).

Aminco DW-2a recording spectrophotometer as described previously [14]. Steroids were added to the sample cuvette in small volumes of ethanol (5–10 μl), and an equal volume of the vehicle alone was added to the reference cuvette.Benzphetamine was added to microsomes dissolved in water. All spectra were corrected for the baseline of equal light absorbance. Microsomal protein concentrations were determined by the method of Lowry *et al.* [24] using bovine serum albumin as the standard.

RESULTS

Testosterone decreased the rates of benzphetamine and benzo[a]pyrene (BP) metabolism by adrenal microsomes but had no effect on the activities of hepatic enzymes (Table 1). 6β-Hydroxytestosterone, in contrast, had no effect on enzyme activity in either tissue. Estradiol decreased benzphetamine demethylation in both adrenal and liver microsomes, but only at high steroid concentrations. Adrenal BP hydroxylase activity was increased by estradiol at concentrations between 5×10^{-6} and 4×10^{-5} M; hepatic BP metabolism was increased only by the lowest concentration of estradiol.

Progesterone produced a concentration-dependent inhibition of adrenal benzphetamine and BP metabolism but had considerably less effect on hepatic metabolism of both substrates (Table 2). 17-Hydroxyprogesterone was a less potent inhibitor of

Table 2. Effects of progesterone and 17-hydroxyprogesterone on adrenal and hepatic benzphetamine demethylase and benzo[a]pyrene hydroxylase activities

Steroid conc (μM)	Benzphetamine demethylation*		Benzo[a]pyrene hydroxylation*	
	Adrenal	Liver	Adrenal	Liver
Control	100	100	100	100
Progesterone				
2	86 ± 8	100 ± 1	94 ± 3	97 ± 5
5	73 ± 9†	96 ± 1	86 ± 6†	95 ± 5
10	65 ± 7†	95 ± 3	74 ± 7†	86 ± 9
40	41 ± 4†	89 ± 12	58 ± 8†	77 ± 6†
17-Hydroxyprogesterone				
2	99 ± 1	101 ± 2	94 ± 4	99 ± 4
5	95 ± 2†	100 ± 1	91 ± 4†	98 ± 2
10	88 ± 3†	102 ± 3	88 ± 3†	93 ± 5
40	67 ± 4†	99 ± 2	76 ± 3†	87 ± 6†

* Values are expressed as mean per cent of control ± S.E.; six to eight determinations per value.
† $P < 0.05$ (vs control).

Table 3. Effects of 11-deoxycorticosterone and 11-deoxycortisol on adrenal and hepatic benzphetamine demethylase and benzo[*a*]pyrene hydroxylase activities

Steroid conc (μM)	Benzphetamine demethylation*		Benzo[*a*]pyrene hydroxylation*	
	Adrenal	Liver	Adrenal	Liver
Control	100	100	100	100
11-Deoxycorticosterone				
2	66 ± 10†	101 ± 2	74 ± 10†	99 ± 1
5	55 ± 8†	101 ± 5	68 ± 11†	100 ± 2
10	46 ± 8†	99 ± 6	55 ± 11†	102 ± 3
40	26 ± 4†	105 ± 4	38 ± 9†	93 ± 6
11-Deoxycortisol				
2	95 ± 3	102 ± 2	96 ± 3	95 ± 5
5	92 ± 3†	101 ± 6	92 ± 3†	100 ± 5
10	83 ± 5†	101 ± 1	85 ± 9†	98 ± 4
40	61 ± 5†	103 ± 3	73 ± 7†	92 ± 5

* Values are expressed as mean per cent of control ± S.E.; six to eight determinations per value.
† $P < 0.05$ (vs control).

adrenal drug metabolism than progesterone and also had little effect on hepatic metabolism.

The presence of the C-21 hydroxyl group did not diminish the inhibitory effects of corticosteroids on adrenal benzphetamine demethylation and BP hydroxylation (Table 3). 11-Deoxycorticosterone was a potent inhibitor of both reactions in adrenal microsomes but had no effect on either reaction in hepatic microsomes. The 17-hydroxylated counterpart of 11-deoxycorticosterone, 11-deoxycortisol, also did not affect hepatic xenobiotic metabolism (Table 3). However, the presence of the 17-hydroxyl group again decreased the inhibitory effects on adrenal BP and benzphetamine metabolism. Thus, 11-deoxycortisol was a less potent inhibitor of adrenal metabolism than 11-deoxycorticosterone.

The presence of a hydroxyl group at the C-11 position had little effect on steroid inhibition of adrenal drug metabolism (Table 4). Corticosterone, which is structurally identical to 11-deoxycorticosterone except for the 11-hydroxyl group, was as potent an inhibitor of adrenal benzphetamine and BP metabolism as 11-deoxycorticosterone. Similarly, the effects of cortisol were at least as great as its 11-deoxy equivalent, 11-deoxycortisol. The 17-hydroxylated corticosteroid, cortisol, was a less potent inhibitor of adrenal drug metabolism than the 17-deoxy compound, corticosterone. Neither cortisol nor corticosterone affected the rates of hepatic BP and benzphetamine metabolism.In contrast to the actions of cortisol, 6β-hydroxycortisol had no effects on adrenal xenobiotic metabolism. Hepatic enzyme activities were also not affected by 6β-hydroxycortisol.

Addition of cortisol or benzphetamine to adrenal microsomes produced a type I difference spectrum.

Table 4. Effects of corticosterone, cortisol and 6 β-hydroxycortisol on adrenal and hepatic benzphetamine demethylase and benzo[*a*]pyrene hydroxylase activities

Steroid conc (μM)	Benzphetamine demethylation*		Benzo[*a*]pyrene hydroxylation*	
	Adrenal	Liver	Adrenal	Liver
Control	100	100	100	100
Corticosterone				
2	82 ± 7†	98 ± 4	79 ± 4†	99 ± 3
5	69 ± 8†	106 ± 4	58 ± 5†	103 ± 3
10	56 ± 8†	102 ± 2	49 ± 3†	105 ± 5
40	33 ± 4†	99 ± 1	29 ± 3†	101 ± 9
Cortisol				
2	93 ± 5	96 ± 2	88 ± 3†	95 ± 6
5	90 ± 7	102 ± 2	83 ± 7†	95 ± 4
10	77 ± 8†	97 ± 2	71 ± 12†	98 ± 3
40	55 ± 7†	101 ± 1	51 ± 9†	95 ± 3
6β-OH-cortisol				
2	100 ± 1	101 ± 1	97 ± 2	99 ± 3
5	102 ± 1	99 ± 2	98 ± 2	100 ± 5
10	103 ± 1	102 ± 1	94 ± 3	96 ± 3
40	102 ± 2	103 ± 1	96 ± 5	96 ± 1

* Values are expressed as mean per cent of control ± S.E.; six to eight determinations per value.
† $P < 0.05$ (vs control).

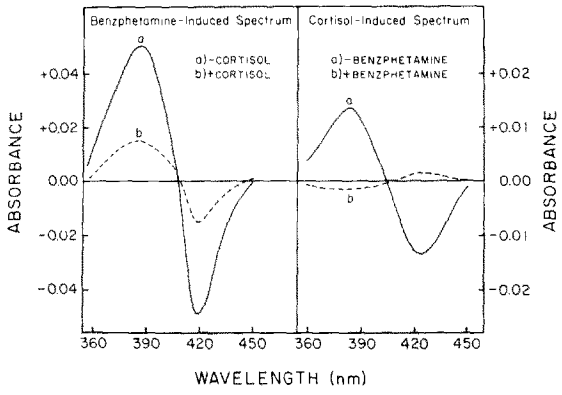

Fig. 1. Cortisol- and benzphetamine-induced difference spectra in adrenal microsomes. Cuvettes contained 1.1 mg of adrenal microsomal protein/ml. On the left, curve a was recorded after addition of 5×10^{-3}M benzphetamine to the sample cuvette and an equal volume of water to the reference cuvette. Curve b was obtained by addition of 5×10^{-3}M benzphetamine to the sample cuvette after adding 5×10^{-5}M cortisol to both the sample and reference cuvettes. On the right, curve a was recorded after addition of 5×10^{-5}M cortisol to the sample cuvette and an equal volume of ethanol to the reference cuvette. Curve b was obtained after addition of 5×10^{-5}M cortisol to the sample with 5×10^{-3} M benzphetamine present in both cuvettes.

The spectra produced by saturating concentrations of each compound are shown in Fig. 1. There was no detectable spectral change produced by 6β-hydroxycortisol. Prior addition of cortisol to adrenal microsomes diminished the magnitude of the benzphetamine-induced spectral change (Fig. 1). Similarly, addition of benzphetamine prevented the cortisol-induced spectrum. 6β-Hydroxycortisol had no effect on the spectra produced by benzphetamine or by cortisol.

DISCUSSION

The results establish that a wide variety of steroid hormones influence the activities of drug-metabolizing enzymes in guinea pig adrenal microsomes. Those steroids which inhibited drug metabolism also produced type I spectral changes in adrenal microsomes and diminished the magnitude of the benzphetamine-induced type I spectrum. We have done Lineweaver–Burk plots for several steroids (cortisol, corticosterone and testosterone) and the results indicate that the inhibition of adrenal xenobiotic metabolism by steroids is competitive in nature. Thus, the steroids are probably interacting with the same species of cytochrome P-450 that is required for drug metabolism.

A relative specificity of steroid interactions with adrenal enzymes is indicated by the failure of most of the steroids to affect hepatic drug metabolism. The same steroids also failed to produce type I spectral changes in hepatic microsomes, and their prior addition to the microsomal preparations did not affect the magnitude of the type I spectrum produced by benzphetamine. Other investigators have reported that some steroids are potent inhibitors of hepatic drug metabolism in rats and mice [25–29], observations which we have confirmed. In the guinea pig, however, hepatic drug-metabolizing enzymes appear to be more substrate-specific than those in other rodents since relatively little, if any, effect was exerted by any of the steroids tested. These observations are consistent with other known species differences in the catalytic properties of hepatic microsomal mixed function oxidases [30, 31].

The effects of testosterone on adrenal drug metabolism in the guinea pig are similar to those reported previously for the mouse [26] and rat [29]. In all three species, testosterone inhibits the microsomal metabolism of xenobiotics. The effects of estradiol, however, appear to be species dependent. In rats and mice, estradiol inhibits adrenal BP hydroxylase activity [26, 29]. In the guinea pig, high concentrations of estradiol decrease benzphetamine demethylase activity but enhance the metabolism of BP. The mechanisms responsible for the increase in BP hydroxylase activity are not known but may involve changes in the profile of BP metabolic products. That possibility is presently being investigated with the use of high pressure liquid chromatography to quantify individual metabolites.

One of the principal objectives of these studies was to determine if any relationship could be established between specific steroid hydroxylases and drug-metabolizing enzymes in adrenal microsomes. We had demonstrated previously that adrenal 21-hydroxylase activity and xenobiotic metabolism were independently controlled by several physiological and pharmacological factors [14–16, 32, 33]. The results of those studies also suggested that different cytochrome P-450 moieties were involved in steroid 21-hydroxylation and drug metabolism. The latter conclusion is also supported by the present studies. The inhibitory effects of corticosteroids on adrenal benzphetamine and benzo[*a*]pyrene (BP) metabolism were independent of whether or not the steroids were 21-hydroxylated. Compounds with 21-hydroxyl groups (11-deoxycorticosterone, 11-deoxycortisol), and which accordingly do not interact with the 21-hydroxylase cytochrome P-450, produced as much or more inhibition than their 21-deoxy analogues (progesterone, 17α-hydroxyprogesterone) which are substrates for 21-hydroxylation. Thus, the species of cytochrome P-450 required for 21-hydroxylation is probably distinct from that (those) catalyzing adrenal xenobiotic metabolism. Similarly, the 11β-hydroxylase enzyme complex appears to be independent of the drug-metabolizing mixed function oxidases since the presence of an 11β-hydroxyl group also did not diminish the inhibitory effects of steroids on adrenal drug metabolism. The latter is not surprising because 11β-hydroxylase is a mitochondrial enzyme while benzphetamine demethylase and BP hydroxylase activities are located in the microsomal fraction.

Our observations suggest that there is some degree of overlapping substrate specificity between the 17α-hydroxylase and the drug-metabolizing mixed function oxidases in adrenal microsomes. Steroids with-

out 17-hydroxyl groups (progesterone, 11-deoxycorticosterone and corticosterone) are more potent inhibitors of adrenal drug metabolism than their 17-hydroxylated counterparts (17-hydroxyprogesterone, 11-deoxycortisol and cortisol). Thus, the ability to interact with 17α-hydroxylase cytochrome P-450 seems to increase the potency of steroids as inhibitors of benzphetamine and BP metabolism.

A very close association between the 6β-hydroxylase and drug-metabolizing enzymes in adrenal microsomes is also indicated by our data. Cortisol and testosterone were potent inhibitors of both benzphetamine and BP metabolism by adrenal microsomes but their 6β-hydroxylated metabolites had no effect on either reaction. We have found similar steroid effects on adrenal metabolism of other xenobiotics, including ethylmorphine, biphenyl and hexobarbital. In addition, cortisol produced a type I spectral change in adrenal microsomes and decreased the size of the benzphetamine-induced spectrum whereas 6β-hydroxycortisol did neither, suggesting that interactions with the 6β-hydroxylase are important for steroid inhibition of drug metabolism. Furthermore, prior addition of benzphetamine to adrenal microsomes prevented the cortisol-induced spectral change, indicating that the two compounds were competing for a common binding site. Previous studies have also established a very close relationship between adrenal 6β-hydroxylase and drug-metabolizing activities as a function of strain differences in guinea pigs [32, 34, 35]. Adrenals from the inbred Strain 2 and Strain 13 guinea pigs have very high rates of drug metabolism and 6β-hydroxylation but 21-hydroxylase activity is similar to that in outbred strains, suggesting common genetic regulation of the 6β-hydroxylase and drug-metabolizing mixed function oxidases. Thus, all of the data are consistent with the hypothesis that at least some of the drug-metabolizing enzymes and the 6β-hydroxylase share a common cytochrome P-450 moiety which is different from the 21-hydroxylase cytochrome P-450.

All of the steroids used in our studies are naturally occurring compounds, produced either by the adrenal cortex or by other steroidogenic tissues. Those steroids secreted by the adrenal gland or serving as intermediates in the production of adrenal secretory products are found in relatively high concentrations within the gland. The results presented in this communication suggest that such endogenous steroids may play an important part in the regulation of adrenal xenobiotic metabolism. We demonstrated previously that ACTH administration to guinea pigs decreased adrenal ethylmorphine demethylase activity without affecting cytochrome P-450 levels [14]. It is possible that the effects of ACTH on adrenal drug metabolism are mediated by an increase in steroidogenesis. If so, adrenal drug-metabolizing activity may, in general, be inversely related to steroidogenic activity. Additional studies are now needed to more fully examine this hypothesis.

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REFERENCES

1. E. R. Simpson and J. I. Mason, *Pharmac. Ther. (B)* **2**, 339 (1976).
2. B. Tamaoki, *J. Steroid Biochem.* **4**, 89 (1973).
3. H. D. Colby and R. C. Rumbaugh, in *Extrahepatic Metabolism of Drugs and Other Foreign Compounds*, (Ed. T. E. Gram). Spectrum Publications, Jamaica, NY, in press.
4. L. W. Wattenberg and J. L. Leong, *J. Histochem. Cytochem.* **10**, 412 (1962).
5. O. Pelkonen, P. Arvela and N. I. Karki, *Acta pharmac. tox.* **30**, 385 (1971).
6. M. R. Juchau, *Archs int. Pharmacodyn. Thér.* **194**, 346 (1971).
7. M. R. Juchau and M. G. Pedersen, *Life Sci.* (Part II), **12**, 193 (1973).
8. P. K. Zachariah and M. R. Juchau, *Life Sci.* **16**, 55 (1975).
9. O. Pelkonen and N. T. Karki, *Biochem. Pharmac.* **24**, 1445 (1975).
10. A. B. Rifkind, S. Bennett, E. S. Forster and M. I. New, *Biochem. Pharmac.* **24**, 839 (1975).
11. D. Kupfer, L. L. Bruggeman and T. Munsell, *Archs Biochem. Biophys.* **129**, 189 (1969).
12. D. Kupfer and S. Orrenius, *Molec. Pharmac.* **6**, 221 (1970).
13. J. W. Greiner, R. E. Kramer, D. A. Robinson, W. J. Canady and H. D. Colby, *Biochem. Pharmac.* **25**, 951 (1976).
14. J. W. Greiner, R. E. Kramer, R. C. Rumbaugh and H. D. Colby, *Life Sci.* **20**, 1017 (1977).
15. D. A. Pitrolo, R. C. Rumbaugh and H. D. Colby, *Drug Metab. Dispos.* **7**, 52 (1979).
16. J. W. Greiner, R. E. Kramer and H. D. Colby, *Prostaglandins* **17**, 587 (1979).
17. T. M. Guenthner, D. W. Nebert and R. H. Menard, *Molec. Pharmac.* **15**, 719 (1979).
18. D. A. Haugen, T. A. van der Hoeven and M. J. Coon, *J. biol. Chem.* **250**, 3567 (1975).
19. P. E. Thomas, A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek and W. Levin, *J. biol. Chem.* **251**, 1385 (1976).
20. C. R. Jefcoate, R. Hume and G. S. Boyd, *Fedn Eur. Biochem. Soc. Lett.* **91**, 41 (1970).
21. S. Isaka and P. F. Hall, *Biochem. biophys. Res Commun.* **43**, 747 (1971).
22. D. W. Nebert and H. V. Gelboin, *J.biol. Chem.* **243**, 6242 (1968).
23. T. Nash, *Biochem. J.* **55**, 416 (1953).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. T. R. Tephly and G. J. Mannering, *Molec. Pharmac.* **4**, 10 (1968).
26. D. W. Nebert, L. L. Bausserman and R. R. Bates, *Int. J. Cancer* **6**, 470 (1970).
27. T. Spencer, *Enzymologica* **43**, 301 (1972).
28. J. Booth, G. R. Keysell and P. Sims. *Biochem. Pharmac.* **23**, 735 (1974).
29. A. D. Rahintula, P. K. Zachariah and P. J. O'Brien, *Br. J. Cancer* **40**, 105 (1979).
30. S. S. Thorgeirsson, S. A. Atlas, A. R. Boobis and J. S. Felton, *Biochem. Pharmac.* **28**, 217 (1979).
31. R. Kato, *Pharmac. Ther.* **6**, 41 (1979).
32. H. D. Colby, R. C. Rumbaugh, M. L. Marquess, P. B. Johnson, M. R. Pope and R. E. Stitzel, *Drug Metab. Dispos.* **7**, 270 (1979).
33. J. W. Greiner, R. C. Rumbaugh, R. E. Kramer and H. D. Colby, *Endocrinology* **103**, 1313 (1978).
34. S. Burstein, B. R. Bhavnani and M. Gut, *J. biol. Chem.* **240**, 2845 (1965).
35. S. Burstein, *Biochem. biophys. Res. Commun.* **26**, 697 (1967).