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KETOCONAZOLE INHIBITION OF PROGESTERONE OXIDATION BY THE RABBIT

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Abstract—Ketoconazole, a known cytochrome P-450 inhibitor, inhibited both progesterone ring hydroxylation and side-chain oxidation to steroidal acids. Progesterone 21 6β- and 16α-hydroxylase activities of rabbit liver microsomes were inhibited 50% by ketoconazole at concentrations between 10^{-5} and 10^{-4} M. Steroid acid formation was similarly inhibited at a 10^{-5} M concentration. Ketoconazole administration to rabbits produced a significant reduction in the urinary excretion of acidic metabolites of [^{3}H]deoxycorticosterone and [^{14}C]progesterone by approximately 50 and 75% respectively. The differential effect of ketoconazole *in vivo* may indicate that more than one acidic metabolite pathway may be operative.

Ketoconazole is an imidazole derivative that has been reported to be a potent inhibitor of steroidogenesis [1]. Steroids provide useful substrates for studies on site-specific hydroxylations catalysed by a variety of cytochromes P-450 acting on a single substrate. Thus, ketoconazole has been shown to inhibit the regio-specific hydroxylation of androstenedione† at the 6β-, 16β-, and 16α-positions by phenobarbital-treated rat liver microsomes [2]. The anti-cytochrome P-450 action of ketoconazole was realized when its antifungal growth properties were attributed to the inhibition of a cytochrome P-450-dependent 14α-demethylation step in the biosynthesis of ergosterol, an essential sterol [3]. This reaction involves the formation of an intermediary carboxylic acid function [4] which resembles the oxidation of the acetyl side-chain of progesterone to a 20-oxo-21-oic acid by rabbit liver microsomes (Fig. 1; [5]). This reaction requires an initial 21-hydroxylation of progesterone to give DOC [6], which is catalysed by a particularly active cytochrome P-450 Form 1 [7]. Further oxidation of the α-ketol side-chain of DOC appears to also involve a microsomal cytochrome P-450-system [8] although the enzymes have not yet been isolated. The rabbit excretes acidic metabolites of progesterone in the urine which have been characterised as having predominantly the 20-oxo-21-oic acid-chain [9]. 6-Hydroxylation is also a major metabolic pathway in the rabbit and results in the excretion of 6-hydroxylated neutral and acidic metabolites [10, 11]. Ketoconazole has been used in the present study to examine its potency as an inhibitor of regio-specific hydroxylations, which are catalysed by distinct cytochrome P-450 isozymes, and to determine its effect on the excretion of steroidal acids.

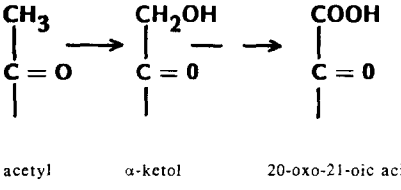

Fig. 1. Side-chain oxidations of progesterone.

MATERIALS AND METHODS

In vitro *experiments*. New Zealand White rabbits (3–4 kg) were starved overnight, and the liver microsomes were prepared as previously described [8]. Incubations were carried out at 37° for 10 min after a 3-min preincubation with liver microsomes (0.1 mg protein/ml), [4-^{14}C]progesterone or non-labelled progesterone (10 nmol in 10 μl methanol) and with or without addition of ketoconazole (20 μl in ethanol). The reaction was started by addition of NADPH (500 μM in 0.1 ml buffer) to give a final volume of 1.0 M potassium phosphate buffer, pH 7.4, and a ketoconazole concentration of 10^{-8} to 10^{-3} M. Incubations were terminated by extraction with ethyl acetate (2 × 5 ml), and after evaporation under a nitrogen stream the products were examined by either TLC in chloroform–ethyl acetate (6:4, v/v) or HPLC. A C_{18}-Ultrasphere ODS (4.5 mm × 25 mm) column was developed with an isocratic methanol–water (65:35, v/v) system at a flow rate of 1 ml/min, conditions which had been optimised for the resolution of the major hydroxy-

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† Nomenclature and abbreviations: 6α-OHP, 6α-hydroxy-4-pregnene-3,20-dione; 16α-OH, 16α-hydroxy-4-pregnene-3,20-dione; DOC (deoxycorticosterone), 21-hydroxy-4-pregnene-3,20-dione; progesterone, 4-pregnene-3,20-dione; testosterone, 17β-hydroxy-4-androstene-3-one; androstenedione, 4-androstene-3,17-dione; pregnenoic acid, 4-pregnene-3,20-dione-21-oic acid; and etienic acid, 4-androstene-3-one-17β-carboxylic acid.

lated metabolites of progesterone, as reported elsewhere [12]. Appropriate steroid standards were run with both systems and detected by their UV absorption. Radio metabolites were detected with a radiochromatogram scanner (Packard Instruments, U.S.A., model 7201) and eluted with methanol.

Acidic metabolites of [4-^{14}C]DOC (4 nmol) were isolated from 30-min incubates of rabbit liver microsomes (1.0 mg protein/ml) conducted as described above. The organic extracts were partitioned with sodium bicarbonate solution (8%, w/v) and the acids reextracted into ethyl acetate at pH 1. Aliquots of the total acids in the extracts were removed for counting in a liquid scintillation counter.

In vivo *experiments*. Rabbits (female, 3.5 to 4 kg) were housed in individual metabolic cages and allowed water and lab chow *ad lib*. [^{3}H]DOC (25 μCi) and [4-^{14}C]progesterone (5 μCi) were dissolved in ethanol (0.5 ml) and diluted with an equal volume of saline immediately before intravenous (i.v.) injection of a marginal ear vein or intraperitoneal (i.p.) injection. Urine was collected for consecutive 24-hr periods and continued until radioactivity levels had declined to background levels, which required about 2 weeks. The same rabbits were then injected i.p. with ketoconazole dissolved in warm peanut oil containing 10% ethanol (2 ml; 50 mg/kg body wt) for 4 consecutive days. On day 5 each rabbit received the same dose of [^{3}H]DOC/[^{14}C]progesterone administered via the same route as previously. The first 24-hr urines from control and ketoconazole-treated rabbits were processed on Amberlite XAD-2 columns [13], and the evaporated methanolic eluates were hydrolysed with Glucurase (Sigma Chemical Co., U.S.A.; 1000 units β-glucuronidase/ml). Hydrolysates were extracted at pH 1 with ethyl acetate (2 × 5 ml), and the acids were partitioned into sodium bicarbonate (8%, w/v) and then back into ethyl acetate at pH 1. Aliquots of the neutral and acidic metabolite extracts were counted in a liquid scintillation counter (Beckman, U.S.A.; LS 3801).

Chemicals and supplies. [1,2-^{3}H]Deoxycorticosterone (40–60 Ci/mmol) and [4-^{14}C]progesterone (54.2 mCi/mmol) were obtained from New England Nuclear, Canada, and stored in methanolic solution. Radioisotopes were counted in 10 ml of PCS–xylene scintillation fluid (1:1, v/v) obtained from Amersham, U.S.A., and Fisher Scientific, U.S.A., respectively. HPLC grade solvents were purchased from Burdick & Jackson, U.S.A., and other solvents and chemicals from Anachemia, Montreal, Canada, or Fisher Scientific, U.S.A. Ketoconazole was a gift from Janssen Pharmaceutica, Ontario, Canada. Non-labelled steroids were supplied by Steraloids, U.S.A., or the Sigma Chemical Co., U.S.A. Outbred New Zealand White rabbits of both sexes were obtained from Ferme Cunicole, Claude Leonard, Quebec, Canada.

RESULTS

Neutral steroid metabolites in vitro. Incubation of progesterone with rabbit liver microsomes for short periods produces monohydroxylated metabolites that can be resolved by HPLC [12]. Figure 2 shows a typical spectrum of progesterone metabolites and the complete inhibition of the regio-specific hydroxylations by incubation in the presence of ketoconazole (10^{-3} M).

The monohydroxylated progesterone metabolites were also resolved by TLC, a procedure which allows a sensitive assay of [^{14}C]progesterone metabolism and has been used to study its reaction kinetics [8]. Figure 3 shows representative radiochromatogram scans of control and ketoconazole-inhibited

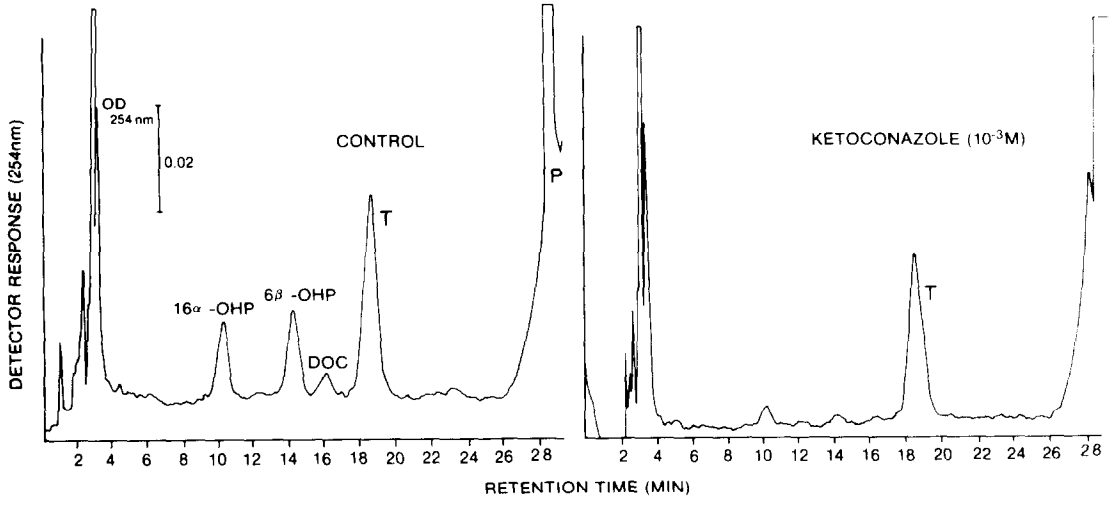

Fig. 2. High performance liquid chromatograms of progesterone metabolites. Progesterone (P) (10 nmol) was incubated with rabbit liver microsomes (0.1 mg protein/ml) and NADPH (500 μM) for 10 min with and without addition of ketoconazole (20 μl ethanolic solution; 10^{-3} M). Testosterone (T) (500 ng) was added to the incubate extracts as internal standard. Extracts were evaporated and then dissolved in 30 μl methanol; 20 μl was injected in duplicate. Boiled microsomes gave the same chromatogam as the ketoconazole-treated microsomes.

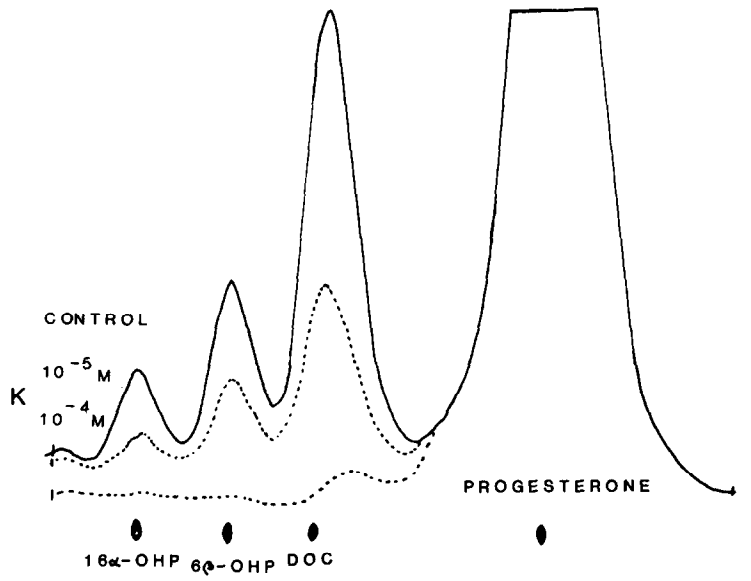

Fig. 3. Radiochromatogram scans of [^{14}C]progesterone metabolites separated by TLC. K = ketoconazole.

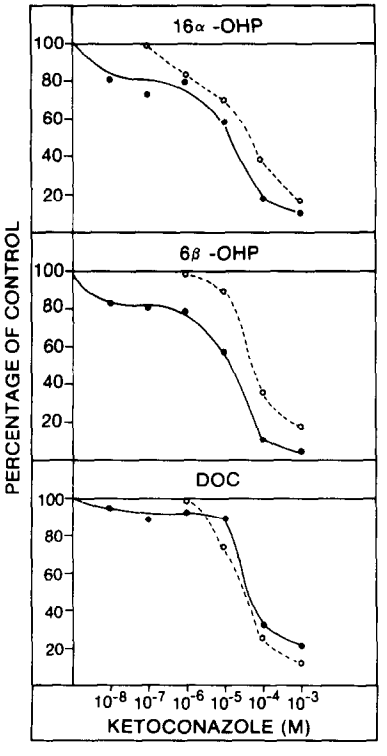

Fig. 4. Dose–response curves for the inhibition of progesterone 21-, 6β- and 16α-hydroxylase activities by liver microsomes. [4-^{14}C]Progesterone (10 nmol) was preincubated for 5 min with microsomes (0.1 mg protein/ml) with and without ketoconazole (10^{-8}–10^{-3} M). Reactions were started by addition of NADPH (500 μM) and terminated after 10 min with ethyl acetate. Neutral metabolites were separated by TLC and analysed as described in Materials and Methods. All measurements were the mean of duplicates, and the two plots depict microsomes from different rabbits. Control values: progesterone 21-hydroxylase: 1.34 and 0.48 nmol DOC formed·min^{-1}·(mg protein)$^{-1}$; 16α-/6β-hydroxylase: 0.5/0.64 and 0.6/1.04 nmol·min^{-1}·(mg protein)$^{-1}$.

metabolism. New Zealand White rabbits show considerable intraspecies variability in their hepatic microsomal content and activity of progesterone 21-hydroxylase [5, 14]. The dose-dependent response curves for the ketoconazole inhibition of individual progesterone hydroxylases shown in Fig. 4 compare hepatic microsomes from two rabbits. Progesterone 21-hydroxylase activities were 1.34 and 0.48 nmol DOC formed·min^{-1}(mg protein)$^{-1}$, and the corresponding 16α-/6β-hydroxylase activities were 0.5/0.64 and 0.6/1.04 nmol·min^{-1}(mg protein)$^{-1}$ respectively. Hepatic microsomes exhibiting low progesterone 21-hydroxylase activities [<0.1 nmol DOC·min^{-1}(mg protein)$^{-1}$] have also been observed [15]. It was of interest to note that in these preparations the metabolism of progesterone to pregnanolone (5β-pregnan-3α-ol-20-one), a ring A reduced metabolite previously characterised [16], was insensitive to the presence of ketoconazole (10^{-3} M), indicating the specificity of ketoconazole for inhibition of monooxygenases.

The dose-dependent curves for the inhibition by ketoconazole shown in Fig. 4 give a 50% inhibition

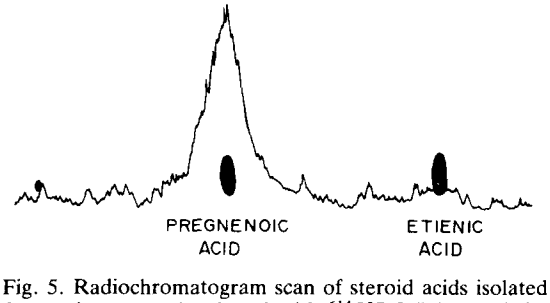

Fig. 5. Radiochromatogram scan of steroid acids isolated from microsomes incubated with [^{14}C]DOC (4 nmol) for 30 min and separated by TLC in ethyl acetate–formic acid (99:1, v/v). Internal standards were detected by their UV absorption.

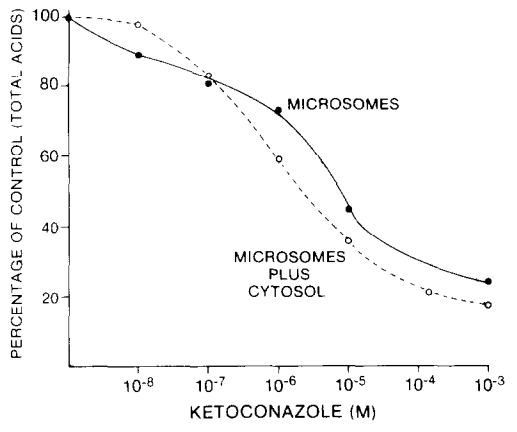

Fig. 6. Dose–response curves for steroid acid formation. [4-^{14}C]DOC (10 nmol) was preincubated for 5 min with liver microsomes (1.0 mg protein/ml) with or without addition of ketoconazole (10^{-8}–10^{-3} M). Cytosol (0.05 mg; 1.28 mg protein/ml) was added to a second set of duplicate experiments. Reactions were started by addition of NADPH (500 μM), and acidic metabolites were partitioned between ethyl acetate and sodium bicarbonate after 30 min and estimated as described in Materials and Methods. The dotted line shows the effect of cytosol. The reaction rate for α-ketol oxidase activity averaged 38 pmol pregnenoic acid formed·min^{-1}·(mg microsomal protein)$^{-1}$ over the 30-min incubation period.

of all three progesterone hydroxylases at a ketoconazole concentration between 10^{-5} and 10^{-4} M.

Acidic steroid metabolites in vitro. The α-ketol side chain of DOC derived by C-21-hydroxylation of progesterone may be oxidised further to a C-21-oic acid (pregnenoic acid) [17]. Figure 5 shows a radiochromatogram of a steroid acid fraction isolated from an incubation of [^{14}C]DOC with rabbit liver microsomes and subjected to TLC in ethyl acetate–formic acid (99:1, v/v). The reaction rate for α-ketol oxidase activity averaged 38 pmol pregnenoic acid formed·min^{-1}·(mg microsomal protein)$^{-1}$ over the 30-min incubation period. Since pregnenoic acid was the only steroid acid of quantitative significance, total acid formation was assessed by solvent partition, without TLC. In Fig. 6, the dose-dependent response curves for ketoconazole inhibition show that 50% inhibition of acid formation occurred at ketoconazole concentrations between 10^{-6} and 10^{-5} M. An unexpected finding was the increased sensitivity to ketoconazole inhibition when small aliquots of cytosol from the same liver were added to the microsomal incubates.

In vivo *metabolism*. Since the *in vitro* studies showed that ketoconazole was a potent inhibitor of both progesterone 21-hydroxylation and α-ketol oxidation to the 21-oic acid, it was of interest to examine the effects of this inhibitor *in vivo*. Mixtures of [^{3}H]DOC and [^{14}C]progesterone were administered to two rabbits via different routes, namely intravenous and intraperitoneal, before and after the intraperitoneal administration of ketoconazole. Each rabbit therefore served as its own control. The first 24-hr urine from each rabbit was incubated with β-glucuronidase to hydrolyse steroid conjugates, and neutral and acidic metabolite fractions were determined by solvent partition. Table 1 shows that ketoconazole reduced the proportion of steroid acid metabolites of [^{14}C]progesterone and [^{3}H]DOC excreted in the first 24-hr urine by approximately 50 and 75% respectively. This was also reflected in the increase in ^{3}H/^{13}C ratios in the acid fraction after ketoconazole treatment. Comparable percentages of the injected radioactivity were excreted before and after ketoconazole treatment, indicating that excretion was not impaired by pharmacological doses of the inhibitor.

DISCUSSION

Our results show that ketoconazole was a potent inhibitor of both ring and side-chain oxidations of progesterone. Although hydroxylations at the 21-, 6β- and 16α-positions are catalysed by different cytochrome P-450 isozymes (cytochrome P-450 Form 1 [18] and two subforms of cytochrome P-450LM3b [7] respectively), they were all inhibited to approximately the same degree by ketoconazole at a concentration between 10^{-5} and 10^{-4} M. Ketoconazole inhibition of the 6β, 16β- and 16α-hydroxylation of androstenedione by phenobarbital-treated rat liver microsomes has been reported to occur with slightly

Table 1. Effect of ketoconazole on the excretion of progesterone metabolites in the urine

Expt. No.	Route of administration		Percentage of radioactivity* Neutral metabolites ^{3}H	Neutral metabolites ^{14}C	Acidic metabolites ^{3}H	Acidic metabolites ^{14}C	% Inhibition of acids ^{3}H	% Inhibition of acids ^{14}C
1.	i.v.	Control	24.6	35.0	63.5	64.3		
		(^{3}H/^{14}C)		(4.7)		(4.8)		
		Ketoconazole-treated	58.4	77.2	30.4	14.3		
		(^{3}H/^{14}C)		(5.6)		(16.0)	52.4	77.8
2.	i.p.	Control	34.7	37.8	48.7	67.8		
		(^{3}H/^{14}C)		(3.7)		(2.3)		
		Ketoconazole-treated	66.3	81.9	22.4	15.3		
		(^{3}H/^{14}C)		(4.2)		(8.2)	54.0	77.4

* Percentage of radioactivity in urine taken for β-glucuronidase hydrolysis. Excretion of radioactivity was comparable in the urines of the control and ketoconazole-treated rabbits and averaged 35.9% of the i.v. dose and 54.2% of the i.p. dose.

higher potency with concentrations between 10^{-7} and 10^{-5} M [2]. Again there were no significant differences between the different regio-specific hydroxylations though some differential inhibition was observed with different imidazoles. The rabbit is unusual compared to other species such as the rat in that the liver microsomes contain an extra endocrine 21-hydroxylase [6, 7] that converts one hormone, progesterone, into a second potential hormone, DOC. Further oxidation of the α-ketol side-chain of DOC *in vitro* with rabbit liver microsomes results in the accumulation of a 20-oxo-21-oic acid (4-pregnene-3,20-dione-21-oic acid) [5]. The α-ketol oxidase has been solubilised and appears also to be a cytochrome P-450 [8]. This would seem to be confirmed by the dose–response curves in Fig. 4 which show that ketoconazole was a potent inhibitor producing 50% inhibition at concentrations between 10^{-6} and 10^{-5} M. The addition of liver cytosol to the microsomes appeared to increase the sensitivity to ketoconazole by an unknown mechanism. Cytosolic fractions of bovine adrenals have, however, also been reported to influence the steroid 21-hydroxylase activity of bovine adrenal microsomes [19]. The metabolism of progesterone to acidic metabolites that are excreted in the urine is a major metabolic pathway in the rabbit [20] and 20-oxo-21-oic acids have been shown to predominate [9]. Table 1 shows that both [^{14}C]progesterone and [^{3}H]DOC were metabolised to acidic metabolites and excreted in the first 24-hr urine. Excretion was not influenced by the route of administration. Ketoconazole pretreatment had a differential effect on the proportions of acidic metabolites excreted being more potent towards the metabolism of [^{14}C]progesterone (77.4 to 77.8% inhibition) than towards [^{3}H]DOC (52.4 to 54.0% inhibition). This may reflect a greater potency of ketoconazole towards the 21-hydroxylation of progesterone compared to its inhibition of the α-ketol oxidase activity. The incomplete inhibition of the conversion of [^{3}H]DOC to acidic metabolites *in vivo* is of further interest in light of the presence of alternative oxidative pathways to the cytochrome P-450-dependent one in rabbit liver microsomes. A cytosolic, cytochrome P-450-independent pathway that converted DOC to a 21-hydroxy-21-oic has been reported [21] and side-chain oxidation of DOC to C-20-carboxylic acids is known to occur [5]. Ketoconazole may prove useful in differentiating these different metabolic pathways.

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REFERENCES

1. D. Feldman, *Endocr. Rev.* **7**, 409 (1986).
2. J. J. Sheets, J. I. Mason, C. A. Wise and R. W. Estabrook, *Biochem. Pharmac.* **35**, 487 (1986).
3. H. Van den Bossche, C. Willensens, W. Cools, P. Marchal and W. Lauwers, *Biochem. Soc. Trans.* **11**, 665 (1983).
4. Y. Aoyama, T. Okikawa and Y. Yoshida, *Biochim. biophys. Acta* **665**, 586 (1981).
5. I. R. Senciall, G. Bullock and S. Rahal, *Can. J. Biochem. Cell Biol.* **61**, 722 (1983).
6. A. C. Dey and I. R. Senciall, *Can. J. Biochem.* **55**, 602 (1977).
7. H. H. Dieter, U. Muller-Eberhard and E. F. Johnson, *Biochem. biophys. Res. Commun.* **105**, 515 (1982).
8. I. R. Senciall, S. Rahal and K. Sethumadhavan, *J. Steroid Biochem.* **23**, 1083 (1985).
9. I. R. Senciall, C. A. Harding and A. C. Dey, *J. Steroid Biochem.* **9**, 385 (1978).
10. J. G. Allen, A. M. Cooke and G. H. Thomas, *J. Endocr.* **40**, 153 (1968).
11. B. A. Knights, A. W. Rogers and G. H. Thomas, *Biochem. biophys. Res. Commun.* **8**, 253 (1972).
12. I. R. Senciall, *J. Steroid Biochem.* **28**, 711 (1987).
13. H. L. Bradlow, *Steroids* **11**, 265 (1968).
14. U. Muller-Eberhard, L. Ghizzon, H. L. Liem, M. New, M. Finlayson and E. F. Johnson, *Ann. N.Y. Acad. Sci.* **458**, 225 (1985).
15. I. R. Senciall, S. Rahal and K. Sethumadhavan, *J. Steroid Biochem.* **23**, 1087 (1985).
16. I. R. Senciall, S. Rahal and A. C. Dey, *J. Steroid Biochem.* **14**, 473 (1981).
17. I. R. Senciall and A. C. Dey, *J. Steroid Biochem.* **9**, 1093 (1978).
18. H. H. Dieter and E. F. Johnson, *J. biol. Chem.* **257**, 9315 (1982).
19. F. I. Chaselow and S. Lieberman, *J. biol. Chem.* **254**, 3777 (1979).
20. A. M. Cooke, A. W. Rogers and G. H. Thomas, *J. Endocr.* **27**, 299 (1963).
21. A. R. Purkaystha, K. O. Martin, A. Goldberg and C. Monder, *J. Steroid Biochem.* **17**, 51 (1982).