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STEROID BIOSYNTHESIS BY A SESTERTERPENE PATHWAY IN THE RAT ADRENAL GLAND *IN VITRO*

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Summary—Rat adrenal gland preparations were incubated with radioactive cholesterol and 23,24-dinor-5-cholen-3β-ol. Steroids were isolated, purified by thin-layer and high performance liquid chromatography and crystallised to constant specific activity. It was found that the rat adrenal gland can utilise 23,24-dinor-5-cholen-3β-ol to produce corticosterone. Also, in contrast to the conversion of cholesterol to corticosterone which occurs in the mitochondrial fraction, the conversion of 23,24-dinor-5-cholen-3β-ol to corticosterone occurs in the microsomal fraction.

It was concluded that the sesterterpene pathway for steroid biosynthesis can function in the rat adrenal gland and that the intermediates are converted to steroid hormones in the microsomal fraction.

INTRODUCTION

Cholesterol is widely considered to be an obligatory intermediate in the biosynthesis of C_{21}, C_{19} and C_{18} steroids, although not all evidence supports this view [1–5]. It has been proposed that steroidogenesis could also occur by a sesterterpene pathway [4]. The hypothesis is that a sesterterpene hydrocarbon (five isoprene units), analogous to squalene in the triterpene pathway, could cyclize to give a compound analogous to lanosterol but with five carbon atoms fewer on the side chain, 23,24,25,26,27-pentanor-8-lanosten-3β-ol (Fig. 1, **I**). This, after demethylation and double bond migration, as in the classical pathway, would give a compound analogous to cholesterol but with five carbon atoms (one isoprene unit) fewer in the side chain, i.e. 23,24-dinor-5-cholen-3β-ol (Fig. 1, **II**) [4].

In support of this hypothesis it has been shown that, 23,24-dinor-5-cholen-3β-ol (Fig. 1, **II**) is converted to 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) and 23,24-dinor-5-cholene-3β,21-diol (Fig. 1, **IV**), and 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) is converted to cortisol and other steroids by bovine adrenal glands *in vitro* [4]. Canine and human adrenal tissues also convert 23,24-dinor-5-cholen-3β-ol to steroid hormones [6, 7]. It has also been shown that radioactive acetate is incorporated *in vitro* into 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) and 23,24-dinor-5-cholene-3β,21-diol (Fig. 1, **IV**) by bovine adrenals [8] and into the 3β,21-diol (Fig. 1, **IV**) by human adrenals [7]. We have proposed that the pregnant mare may use the sesterterpene pathway for steroidogenesis [6] and have shown that 23,24-dinor-5-cholen-3β-ol is converted to dehydroepiandrosterone by the fetal horse gonad *in vitro* [9]. Recently it has been shown that 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) is converted to cortisol *in vivo* by the guinea-pig [10]. In addition 23,24-dinor-5-cholen-3β-ol (Fig. 1, **II**) occurs among the sterols of marine invertebrates [11].

Evidence is now presented to demonstrate that the rat adrenal can utilise 23,24-dinor-5-cholen-3β-ol (Fig. 1, **II**) to produce corticosterone. Also, in contrast to the conversion of cholesterol to corticosterone which occurs in the adrenal mitochondrial fraction, the conversion of 23,24-dinor-5-cholen-3β-ol to corticosterone occurs in the microsomal fraction.

EXPERIMENTAL

23,24-Dinor-[7α-^{3}H]5-cholen-3β-ol (specific activity [sp. act.] 489 GBq/mmol) was synthesised as previously described [6]. [4-^{14}C]Cholesterol (sp. act. 1.96 GBq/mmol was purchased from Amersham International plc, Amersham, Buckinghamshire. The synthesis of non-radioactive 23,24-dinor-5-cholene-3β,20-diol and 23,24-dinor-5-cholene-3β,21-diol has been described previously [4]. Solvents (analytical grade), 2,5-diphenyloxazole (PPO) and 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP) were purchased from BDH Ltd, Poole, Dorset, and silica gel was obtained from E. Merck, Darmstadt, West Germany. NADPH, $NADP^+$ and NAD^+ were the products of Sigma Chem. Co. Ltd, Poole, Dorset. High performance liquid chromatography (HPLC) was carried out using a radial compression C-18, 10 μM column from Waters Associates, Northwich, Cheshire. Radioactivity was determined on a Tracerlab

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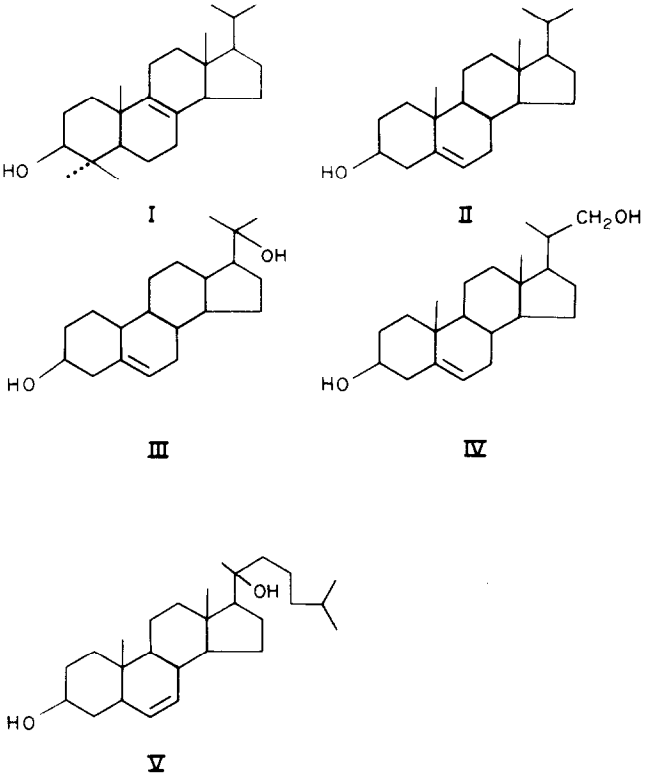

Fig. 1. **I** 23,24,25,26,27-Pentanor-8-lanosten-3β-ol. **II** 23,24-Dinor-5-cholen-3β-ol. **III** 23,24-Dinor-5-cholene-3β,20-diol. **IV** 23,24-Dinor-5-cholene-3β,21-diol. **V** 5-Cholestene-3β,20α-diol.

Corumatic 200 liquid scintillation counter. Samples (300–400 μg) were weighed on a Cahn 26 Automatic Electrobalance and dissolved in 10 ml of scintillation fluid (PPO, 6 g; POPOP, 0.75 g; toluene, 1000 ml), at least 10,000 cpm were collected for each sample. The counting efficiency for tritium and carbon-14 was 30 and 70% respectively. The cross over of carbon-14 in the tritium channel was 10% and tritium in the carbon-14 channel was 0.5%. Procedures for acetylation and hydrolysis of steroids were as described by Bush[12].

Thin-layer chromatography (TLC) was carried out on 0.25 mm layers of silicagel HF 254/366. The solvent systems used were: toluene–ethyl acetate ((A) 1:1, v/v); chloroform–acetone ((B) 4:1, v/v); diethyl ether–acetone ((C) 9:1, v/v); dichloromethane–ethyl alcohol ((D) 19:1, v/v); chloroform (E); cyclohexane–ethyl acetate ((F) 3:2, v/v); dichloromethane–acetone ((G) 19:1, v/v).

Norwegian hooded rats (200–300 g) were killed by cervical dislocation. Rat adrenals (300 mg) were homogenised by hand in 2 ml Krebs–Ringer bicarbonate buffer (pH 7.4) using a glass homogeniser with a Teflon pestle, then incubated at 37°C for 3 h in 5 ml of buffer containing 23,24-dinor-[7α-^{3}H]5-cholen-3β-ol (444 kBq, 2.8 μg) in propylene glycol (50 μl), NADPH (0.250 mmol/l), NAD^+ (0.250 mmol/l) and glucose (11 mmol/l) and gassed with oxygen–carbon dioxide 95:5.

In the experiments with ACTH added *in vitro*, neither substrate, cholesterol nor 23,24-dinor-5-cholen-3β-ol, was efficiently taken up by chopped adrenal tissue. Accordingly we adopted the following preincubation procedure. The adrenal glands were quartered and divided into two portions (approx 300 mg each) and preincubated at 37°C for 40 min. One portion was preincubated in 5 ml Krebs–Ringer bicarbonate buffer pH 7.4, gassed with oxygen–carbon dioxide 95:5, and the other in 5 ml of the gassed buffer containing ACTH (25 i.u. Acthar Corticotrophin, Armour Pharmaceutical Co.). The supernatants were removed, the tissue homogenised in 2 ml of buffer as described above, then incubated at 37°C for 3 h in 5 ml of buffer containing 23,34-dinor- [7α-^{3}H]5-cholen-3β-ol (444 kBq, 2.8 μg) and [4-^{14}C]cholesterol (185 kBq, 365 μg) in propylene glycol (50 μl), NAD^+ (1 mmol/l) and $NADP^+$ (1 mmol/l) and gassed with oxygen–carbon dioxide 95:5.

Adrenal glands (0.7 g) were homogenised as described above in sucrose (5 ml, 0.25 mol/l). The homogenate was centrifuged at 800 ***g*** for 10 min and the supernatant again centrifuged at 10,500 ***g*** for 25 min. The residue obtained was washed by dispersing it in sucrose (5 ml, 0.25 mol/l) and centrifuged first for 10 min at 800 ***g*** (when the residue was discarded) and then for 15 min at 10,500 ***g***. This washing procedure was repeated and the 10,500 ***g*** residue finally obtained

was dispersed in 1 ml of sucrose (0.25 mol/l) containing calcium chloride (0.01 mol/l). The supernatant from the initial 10,500 *g* centrifugation was centrifuged at 105,000 *g* for 90 min and the pellet dispersed in sucrose (1 ml, 0.25 mol/l) containing calcium chloride (0.01 mol/l). The homogenisations and centrifugations were carried out at 4°C. The dispersed residues obtained at 10,500 *g* (referred to as the mitochondrial fraction) and 105,000 *g* (referred to as the microsomal fraction) were incubated at 37°C for 2 h in phosphate buffer (0.1 mol/l, pH 7.4, 2 ml) containing 23,24-dinor-[7α-^{3}H]5-cholen-3β-ol (740 kBq, 4.8 μg) and [4-^{14}C]cholesterol (370 kBq, 729 μg) in propylene glycol (50 μl), magnesium chloride (4 mmol/l), glucose-6-phosphate (6 mmol/l), $NADP^+$ (0.7 mmol/l) and glucose-6-phosphate dehydrogenase (0.2 unit/ml).

Incubations were extracted with chloroform (3 × 25 ml). The combined extracts were washed with water (2 × 10 ml) and the solvent removed under reduced pressure. The residue was dried by azeotropic distillation with ethyl alcohol. Carrier steroids (0.25 μmol) were added to the extracts which were chromatographed in system A, acetylated and rechromatographed in system B, which separates corticosterone, 11-dehydrocorticosterone, deoxycorticosterone acetates and a fraction containing both 23,24-dinor-5-cholene-3β,20-diol 3-acetate and 23,24-dinor-5-cholene-3β,21-diol diacetate. Corticosterone acetate was further purified by sequential chromatography in systems A, C, D and A; 11-dehydrocorticosterone acetate in systems D and A and deoxycorticosterone acetate in systems A and C. 23,24-Dinor-5-cholene-3β,20-diol 3-acetate was separated from 23,24-dinor-5-cholene-3β,21-diol diacetate in system E, and was purified further by TLC in system F and then by HPLC using 10% water in methanol as the mobile phase. 23,24-Dinor-5-cholene-3β,21-diol diacetate was purified further by TLC in system G and then by HPLC using methanol as the mobile phase. Carrier steroid (150 μmol) was added to a portion of the extracts from the final chromatograms and crystallised to constant specific activity, which was considered to be achieved when the values for three consecutive crops agreed to within ±5%. The sp. act. are reported as means ± standard deviations (SD).

RESULTS

Rat adrenal homogenates were incubated with 23,24-dinor-[7α-^{3}H]5-cholen-3β-ol (Fig. 1, **II**), and corticosterone was isolated and crystallised to constant specific activity (27 ± 0.4 dpm/μmol). The crystals, obtained in this way, were combined, acetylated and again crystallised to constant activity (29 ± 1.1 dpm/μmol). From a second experiment the corresponding specific activities were 97 ± 0.9 and 100 ± 1 dpm/μmol, for corticosterone and its acetate respectively.

When rat adrenal homogenates were incubated with 23,24-dinor-[7α-^{3}H]5-cholen-3β-ol and [4-^{14}C]-cholesterol the resulting corticosteroids were labelled with tritium and carbon-14 (Table 1). Following exposure to ACTH *in vitro*, the tritium/carbon-14 ratio in the products was lower than in the experiments without exposure to ACTH. This demonstrates that some distinction was made between 23,24-dinor-5-cholen-3β-ol and cholesterol on stimulating with ACTH and suggests that these substrates were being utilised by different side chain cleaving enzymes.

To test this possibility microsomal and mitochondrial preparations of rat adrenal glands were each incubated with a mixture of 23,24-dinor-[7α-^{3}H]5-cholen-3β-ol and [4-^{14}C]cholesterol. Table 2 shows that the corticosteroid products from the mitochondrial incubation were labelled only with carbon-14 and therefore were derived from cholesterol, but not from 23,24-dinor-5-cholen-3β-ol. Those from the microsomes were labelled only with tritium, indicating that they were derived from 23,24-dinor-5-cholen-3β-ol and not from cholesterol. These results also suggest that, at least with respect to the enzymes involved in the removal of the side chains, there was little or no cross contamination between the microsomal and mitochondrial fractions.

Table 1. Identification of corticosterone, 11-dehydrocorticosterone and deoxycorticosterone as products of the incubation of 23,24-dinor-5-cholen-3β-ol and cholesterol with homogenates of rat adrenal glands

		Mean specific activities of crops dpm/mmol (SD)		Isotope ratio (^{3}H/^{14}C)	
Experiment	Steroid isolated	^{3}H	^{14}C	Product	Substrate
1. Control	Corticosterone acetate	1.2×10^5 (0.01)	4.2×10^4 (0.07)	2.9	3.0
ACTH	Corticosterone acetate	5.8×10^4 (0.16)	3.9×10^4 (0.06)	1.5	3.0
2. Control	Corticosterone acetate	3.3×10^5 (0.07)	1.4×10^5 (0.02)	2.4	2.8
ACTH	Corticosterone acetate	2.3×10^5 (0.07)	2.0×10^5 (0.03)	1.2	2.8
Control	11-Dehydrocorticosterone acetate	2.0×10^5 (0.05)	9.0×10^4 (0.17)	2.2	2.8
ACTH	11-Dehydrocorticosterone acetate	6.1×10^4 (0.19)	5.2×10^4 (0.11)	1.2	2.8
Control	Deoxycorticosterone acetate	5.3×10^5 (0.01)	2.3×10^5 (0.02)	2.3	2.8
ACTH	Deoxycorticosterone acetate	2.8×10^5 (0.07)	2.3×10^5 (0.02)	1.2	2.8

Rat adrenal glands were quartered and preincubated at 37°C for 40 min in Krebs–Ringer bicarbonate with and without the addition of ACTH. The supernatants were removed and the tissue was homogenised and incubated in Krebs–Ringer bicarbonate buffer containing 23,24-dinor-[7α-^{3}H]5-cholen-3β-OL. (444 kBq, 2.8 μg) and [4-^{14}C]cholesterol (185 kBq, 365 μg), NAD^+ (1 mmol/l) and $NADP^+$ (1 mmol/l) at 37°C for 3 h.

Table 2. Identification of corticosterone and deoxycorticosterone as products of the incubation of 23,24-dinor-5-cholen-3β-ol and cholesterol with mitochondria and microsomes of rat adrenal glands

Experiment	Steroid isolated	Mean specific activities of crops dpm/mmol (SD) ^3H	^{14}C
1. Microsomes	Corticosterone	9.0×10^4 (0.32)	—
	Acetylated, recrystallised-corticosterone acetate	9.2×10^4 (0.11)	—
	Deoxycorticosterone acetate	1.6×10^5 (0.05)	—
	Hydrolysed, non-radioactive steroid added and recrystallised-deoxycorticosterone	6.7×10^4 (0.18) (calculated 6.7×10^4)	—
Mitochondria	Corticosterone	—	5.4×10^5 (0.09)
	Acetylated, recrystallised-corticosterone acetate	—	5.6×10^5 (0.06)
	Deoxycorticosterone acetate	—	1.3×10^5 (0.08)
2. Microsomes	Corticosterone	5.1×10^4 (0.08)	—
	Acetylated, recrystallised-corticosterone acetate	5.4×10^4 (0.06)	—
	Deoxycorticosterone acetate	5.5×10^4 (0.07)	—
	Hydrolysed, non-radioactive steroid added and recrystallised-deoxycorticosterone	2.6×10^4 (0.05)	—
Mitochondria	Corticosterone	—	3.1×10^5 (0.01)
	Acetylated, recrystallised-corticosterone acetate	—	3.1×10^5 (0.05)
	Deoxycorticosterone acetate	—	1.3×10^5 (0.02)
	Hydrolysed, non-radioactive steroid added and recrysallised-deoxycorticosterone	—	5.8×10^4 (0.01) (calculated 5.9×10^4)

Rat adrenal gland mitochondrial and microsomal preparations were incubated in phosphate buffer (0.1 mol/l, pH 7.4) containing 23,24-dinor-[7α-^{3}H]-cholen-3β-OL (740 kBq, 4.8 μg), [4-^{14}C]cholesterol (370 kBq, 729 μg), magnesium chloride (4 mmol/l), glucose-6-phosphate (6 mmol/l), $NADP^+$ (0.7 mmol/l) and glucose-6-phosphate dehydrogenase (0.2 unit/ml) at 37°C for 2 h.

The finding that rat adrenal microsomes convert 23,24-dinor-5-cholen-3β-ol to corticosteroids was confirmed by incubating a further preparation of microsomes with 23,24-dinor-[7α-^{3}H]5-cholen-3β-ol, in the presence of NADPH (1 mmol/l) and NAD^+ (1 mmol/l) as cofactors (Table 3).

Adrenal mitochondria produced some polar metabolites from 23,24-dinor-5-cholen-3β-ol. Two of these were identified as 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) and 23,24-dinor-5-cholene-3β,21-diol (Fig. 1, **IV**). 23,24-Dinor-5-cholene-3β,20-diol was isolated and crystallised to constant specific activity (42 ± 1.7 dpm/μmol). The crystals obtained in this way were combined, acetylated and again crystallised to constant activity (38 ± 1.8 dpm/μmol). From a second experiment the corresponding specific activities were 730 ± 20 and 740 ± 16 dpm/μmol for 23,24-dinor-5-cholene-3β,20-diol and 23,24-dinor-5-cholene-3β,20-diol 3-acetate respectively. 23,24-Dinor-5-cholene-3β,21-diol was isolated and crystallised to constant specific activity (250 ± 7 dpm/μmol) and the crystals obtained were combined and acetylated, giving 23,24-dinor-5-cholene-3β,21-diol diacetate, which was crystallised to constant specific activity (260 ± 7 dpm/μmol). The possibility that these diols might also be produced on incubation with the microsome fraction was investigated, but the carrier steroids recovered were not radioactive.

DISCUSSION

The possibility that steroids could be biosynthesised by a pathway other than that through cholesterol has been a recurring idea in the literature [1–5]. Diedrichsen *et al.*[13] have studied the incorporation of radioactive acetate into corticosteroids by rat adrenal glands *in vitro* and concluded that cholesterol is not an obligatory intermediate. They found that, when the conversion of desmosterol to cholesterol was inhibited

Table 3. Identification of corticosterone, 11-dehydrocorticosterone and deoxycorticosterone as products of the incubation of 23,24-dinor-5-cholen-3β-OL with microsomes of rat adrenal glands

Steroid isolated	Mean specific activities of crops dpm/mmol (SD)
Corticosterone acetate	4.20×10^5 (0.03)
Hydrolysed, non-radioactive steroid added and recrystallised-corticosterone	1.14×10^5 (0.02) (calculated 1.25×10^5)
11-Dehydrocorticosterone acetate	1.17×10^6 (0.01)
Hydrolysed, non-radioactive steroid added and recrystallised-11-dehydrocorticosterone	1.72×10^5 (0.05)
Deoxycorticosterone acetate	2.04×10^6 (0.03)
Non-radioactive steroid added, hydrolysed and recrystallized-deoxycorticosterone	7.64×10^5 (0.02) (calculated 8.17×10^5)

Rat adrenal gland microsomes were incubated in phosphate buffer (0.1 mol/l, pH 7.4) containing 23,24-dinor-[7α-^{3}H]5-cholen-3β-OL (740 kBq, 4.8 μg), NADPH (1 mmol/l) and NAD^+ (1 mmol/l) at 37°C for 2 h.

with Triparanol (1-(*p*-diethylaminoethoxyphenyl)-1-(*p*-tolyl)-2-(*p*-chlorophenyl)-ethanol), the specific activity of, for example, corticosterone was decreased only slightly, whereas incorporation of acetate into cholesterol was blocked. These workers therefore proposed that desmosterol is converted to steroid hormones directly. However, our results with homogenates and microsomes from rat adrenal glands suggest that the proposed sesterterpene pathway may be contributing to this non-cholesterol steroid production. Also, since the enzymes concerned with sterol biosynthesis are present in the microsomal fraction [14], it seems possible that 23,24-dinor-5-cholen-3β-ol (Fig. 1, **II**) could be both produced and utilised by the microsomes to make steroid hormones, perhaps in a series of concerted reactions on a multienzyme complex [15].

The observation that 23,24-dinor-5-cholen-3β-ol (Fig. 1, **II**) was converted to 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) and 23,24-dinor-5-cholene-3β,21-diol (Fig. 1, **IV**) in the mitochondrial fraction may explain our previous results that radioactive acetate was incorporated into these diols but not into 23,24-dinor-5-cholen-3β-ol by bovine and human adrenal gland slices [7, 8]. It is possible that acetate was incorporated into 23,24-dinor-5-cholen-3β-ol in the microsomes and, while some of this was converted to steroids, the remainder was taken up by the mitochondria, hydroxylated at C_{20} and C_{21} and not further metabolised.

Our observation that 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) was not converted to steroid hormones by rat adrenal mitochondria confirmed the findings of Burstein *et al.*[16] on the reaction of 20-hydroxylated steroids with bovine adrenal mitochondrial preparations. They found that 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) was not converted to steroid hormones, whereas 5-cholestene-3β,20α-diol (Fig. 1, **V**) underwent the expected side chain cleavage, forming pregnenolone and progesterone. Interestingly, some progesterone (4%) was formed from 24-nor-5-cholene-3β,20α-diol [16], indicating some side chain cleavage, possibly due to the slightly longer ethyl side chain.

Morisaki *et al.*[17] studied pregnenolone formation, from various precursors including 23,24-dinor-5-cholen-3β-ol using a purified bovine adrenal cytochrome P-450 side chain cleaving enzyme preparation. The yield of pregnenolone, measured by gas chromatography–mass spectrometry, from 23,24-dinor-5-cholen-3β-ol was only 7% of the yield produced on incubation of cholesterol. They concluded that a 5–10 carbon side chain was required for the cleavage reaction to occur to any extent. In contrast Arthur *et al.*[18] in a similar study using mitochondria from bovine and rat adrenal glands, concluded that 23,24-dinor-5-cholen-3β-ol was as effective a substrate as cholesterol for pregnenolone production. These authors used a radio-immunoassay for measurement of the pregnenolone formed. Any 23,24-dinor-5-cholene-3β,20-diol (Fig. 1, **III**) formed might have cross-reacted with the antibody, interfered with the assay and given an apparent increase in the pregnenolone formed as the incubation proceeded.

It is generally considered that the major site of 21-hydroxylation is in the microsomal fraction and of 11β-hydroxylation is in the mitochondria. However, these enzyme activities have been reported in other sites throughout the cell. For example, it has been shown that microsomes from the zona fasciculata/reticularis of the rat adrenal cortex can produce corticosterone from endogenous precursors and from added progesterone, showing both 11β- and 21-hydroxylase activities to be present [19]. Also, 21-hydroxylase activity has been found in mitochondrial preparations [16, 20, 21] and 3β-hydroxysteroid dehydrogenase/isomerase activity in both organelles [20, 22]. Our observation that rat adrenal microsomes produced corticosterone from 23,24-dinor-5-cholen-3β-ol also demonstrates that these preparations contained both 11β- and 21-hydroxylase activities as well as 3β-hydroxysteroid dehydrogenase/isomerase activity and the enzymes required to remove the side chain methyl group. Similarly, the production of corticosterone from cholesterol by the mitochondria demonstrates that these preparations have 21-hydroxylase and 3β-hydroxysteroid dehydrogenase activities as well as the expected 11β-hydroxylase and side chain cleaving activities.

The occurrence of 11β-hydroxylase activity in microsomes, and 21-hydroxylase activity in mitochondria, is generally attributed to cross contamination of these fractions during their preparation. However, if there are two pathways for steroidogenesis, and they function separately in the two different intracellular compartments, the full complement of enzymes would be required in each organelle. Possibly the cholesterol pathway functions in one zone of the adrenal gland and the sesterterpene pathway functions in another. Thus, mitochondria from the cells of one zone may use the cholesterol pathway while microsomes from another zone may use the sesterterpene pathway, perhaps to produce one particular steroid.

In conclusion, this study demonstrates that the microsomal fraction from rat adrenal glands can utilise a proposed sesterterpene pathway intermediate, 23,24-dinor-5-cholen-3β-ol, to produce steroid hormones.

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