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ORGANIZATION OF ANDROGEN BIOSYNTHESIS IN THE TESTIS

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SUMMARY

Factors involved in migration of pregnenolone and progesterone from the mitochondria to the endoplasmic reticulum, and in the organization of enzymes involved in androgen biosynthesis in the reticulum have been studied. Pregnenolone and progesterone were differentially concentrated in the endoplasmic vesicle by systems with high capacity and low K_a compared with target organ receptors. The differential binding was reduced by mild trypsin treatment or phospholipase C but only slightly by phospholipase A.

Under similar conditions all three enzymes markedly reduced 3β- and 17β-dehydrogenases; 17α-hydroxylase and 17,20-lyase activities fell to unmeasurable levels. This was associated with the transfer of NADPH-cyt c reductase activity from the microsomal pellet to the cytosol.

A study of the 17α-hydroxylase and 17,20-lyase reactions using [^{3}H]-progesterone and [^{14}C]-17-hydroxyprogesterone indicated that freely diffusible 17-hydroxyprogesterone was not an obligatory intermediate but that the two steps involved consecutive hydroxylation type transfers to an enzyme-bound intermediate.

The overall picture is that of pregnenolone being concentrated and undergoing conversion to progesterone at the outer microsomal surface, progesterone then associating with a cyt-P-450 complex within the membrane which catalyzes consecutive mixed function oxidase reactions, after which the androstenedione formed diffuses outward, partially undergoing 17β-reduction at the outer surface.

INTRODUCTION

For many years it has been recognized that most of the enzymes involved in the biosynthesis of steroid hormones are associated with intracellular membranes. Much work on these enzymes was done on the assumption that a well prepared homogenate would result in membrane fragments being distributed in a uniform liquid phase. Electron micrograms of these fractions, however, revealed that both mitochondrial and microsomal suspensions involved two or more liquid phases separated by membranes which either had remained intact, as in the case of mitochondria and lysosomes, or had largely re-formed into intact vesicles after rupture of the original structure, as in the case of the endoplasmic reticulum. This compartmentalization, therefore, of the enzymes of steroid biosynthesis could play an important role in the process of biosynthesis.

Moreover, investigators had early discovered that certain enzymes were associated with the mitochondria while others which played earlier and later roles in steroid hormone biosynthesis were incorporated into the endoplasmic reticulum. What was responsible for the orderly circulation of intermediates? We have been seeking answers to this question and to the problem of how the enzymes associated with the endoplasmic reticulum are organized on the membrane surfaces. These are not unrelated problems because a mechanism which concentrates a substrate in the environment of an enzyme will affect the rate of reaction; in fact, binding to the active site of an enzyme could account for concentration of substrate.

A number of elegant studies have been done on the localization of mitochondrial enzymes of the adrenal cortex and testes [1, 2]. These have shown that the various components of the mitochondrial hydroxylating systems are located in the inner membrane and the inner fluid compartment.

A. Evidence of specific binding

We became interested in the organization of the microsomal enzymes when we found that incubation of a mixture of [^{3}H]-progesterone and [^{14}C]-17-hydroxyprogesterone with mouse testis microsomes in the presence of adequate cofactors led to the formation of androstenedione and testosterone containing a higher proportion of the progesterone label than the original mixture, rather than less [3]. This was shown to be related to progesterone being concentrated in the microsomes relative to 17-hydroxyprogesterone. The concentrations of the steroids in the microsomal vesicles rather than in the surrounding medium seemed to determine the relative rates of 17α-hydroxylation and 17,20-lyase action.

The relative concentration of progesterone was shown to be due to its differential binding in the microsomes rather than any specific binding of 17-hydroxyprogesterone by the cytosol. In fact, 17-hydroxyprogesterone, androstenedione and testosterone seemed to diffuse readily between the microsomes

Table 1. Distribution of [^{14}C]-pregnenolone and [^{3}H]-progesterone between organelles and cytosol, nmol/mg protein

	15 min		30 min	
	[^{14}C]-Pregnenolone	[^{3}H]-Progesterone	[^{14}C]-Pregnenolone	[^{3}H]-Progesterone
Mitochondria	2·76	1·24	2·52	1·10
Cytosol	0·72	1·18	0·72	1·04
Ratio M/C	3·83	1·05	3·50	1·06
Microsomes	5·24	2·17	5·68	2·36
Cytosol	0·57	1·12	0·51	0·99
Ratio M/C	9·19	1·94	11·14	2·38

Time of incubation at 34°C under N_2.
Each tube contained 20 mg testes equivalent and 2 nmol of each steroid in 1 ml.
At end of incubation tubes centrifuged 105,000 *g* for 60 min.
Pellets corrected for cytosol by lactic dehydrogenase.

and medium [4]. Progesterone did not bind to the testicular mitochondria, so that this steroid would tend to concentrate in the endoplasmic reticulum.

While some investigators have demonstrated that conversion of pregnenolone to progesterone can take place in the mitochondria [5], the majority of evidence indicates that this reaction largely takes place in the endoplasmic reticulum [6, 7]. The major intermediate transferred from the mitochondria would, therefore, be pregnenolone. We investigated the differential distribution of pregnenolone between mitochondria, microsomes and cytoplasm, correcting for contamination of one fraction by the other through the use of marker enzymes. While pregnenolone, unlike progesterone, does concentrate in the mitochondria relative to the cytosol at an approximate ratio of 2·5:1 it concentrates in the microsomes at a ratio of approximately 10:1 (Table 1). The flow of pregnenolone synthesized in the mitochondria would, therefore, be to the endoplasmic reticulum as represented by the microsomal fraction.

B. Nature of the specific binding systems

Before turning to a consideration of the type of compounds which are responsible for the differential distribution of pregnenolone and progesterone between the microsomal fraction and the cytosol it may be helpful to review briefly the structure of the membranes of the endoplasmic reticulum, and therefore of the major portion of the microsomal fraction. Cells actively synthesizing and secreting steroid hormones are filled with smooth endoplasmic reticulum in tubular and vesicular arrangement together with some endoplasmic reticulum to which ribosomes are attached ("rough reticulum"). In highly magnified electron micrographs the walls themselves are seen to be complex, consisting of electron-dense outer and inner surfaces with an intervening electron-transparent zone. The outer and inner faces differ in appearance. Chemical studies indicate that the membrane consists of a typical phospholipid bilayer having proteins associated with, and interposed among, the lipid molecules. The endoplasmic membrane differs from the plasma membrane of the cell wall in being much lower in cholesterol and neutral lipids.

When the endoplasmic reticulum is ruptured during homogenization the fragments round up into vesicles, apparently with the inner face of the reticular structure forming the inner face of the vesicle; at least, where rough reticulum is ruptured the ribosomes are always attached to the outer and not the inner surface of the vesicle. These are the structures, together with some lysosomes and lipid droplets, which make up the microsomal fractions we have studied.

Table 2 indicates that the binding mechanisms for both pregnenolone and progesterone have high capacity and low association constants. In the case of progesterone the capacity exceeded the saturating substrate concentration for 17α-hydroxylase and could not, therefore, simply be saturation of the active site of the enzyme. This could not be determined for 3β-hydroxysteroid dehydrogenase because the limit of solubility of pregnenolone in buffer is reached before the enzyme system is saturated.

We have attempted to use enzymes to determine the types of compound involved in the differential binding and in the associated enzymic reactions. In view of the role of phospholipids in the endoplasmic membrane, we studied the influence of phospholipases A and C on both differential binding of pregnenolone to mouse testis microsomes and on its conversion

Table 2. Distribution of pregnenolone and progesterone between microsomes and sucrose-buffer

Substrates						
Pregnenolone (nmol/ml)	Progesterone (nmol/ml)		Microsomes		Supernatant	
		(A)*	(B)*	(B/A)	(C)*	(C/A)
1	10	0·10	0·26	2·6	0·04	0·4
5	10	0·50	1·24	2·48	0·21	0·42
10	10	1·00	2·74	2·74	0·39	0·39

* A, B and C are ratios of pregnenolone–progesterone.
Incubation 15′ under N_2, no cofactors.

Table 3. Effects of phospholipases on binding of pregnenolone and 3β-dehydrogenase activity (per cent of control suspension)

	Plase* A		Plase C	
	Bound	3βDHase	Bound	3βDHase
Cont. suspen.	100	100	100	100
Plase „	96	15	59	24
Cont. pellet	109	100	96	99
Plase „	77	0	59	22
Plase super.	11	6	6	0

* Plase = phospholipase.

to progesterone (Table 3). Only phospholipase C had a marked effect on binding, although there was some solubilization of the binding factor by phospholipase A. Both phospholipases A and C, however, markedly reduced 3β-hydroxy-dehydrogenase. The results with phospholipase A differed from those of Mancino *et al.* possibly because we activated the enzyme with Ca^{2+} and incubated at a higher temperature [8].

Electron micrographs of microsomal pellets after treatment with each phospholipase are illustrated in Fig. 1. Phospholipase A appeared to cause a loss of organized structure. (This was associated with some loss of protein from the pellet). Vesicular structure seemed to be preserved after treatment with phospholipase C but the vesicular compartment had become filled with material which formed electron-dense deposits with the heavy metal stains.

These results indicated that splitting off of phosphorylated bases with formation of diglycerides led to significant decreases in binding and 3β-ol-dehydrogenase activity. Apparently organization of the membrane was essential to the enzymic activity but of secondary importance to the differential binding.

Mild treatment with trypsin (15 min at 0–1°C) caused a marked decrease in both binding and enzyme activity (Tables 4 and 5) although this treatment actually increased glucose-6-phosphatase activity which has been shown to be associated with the inner surface of the microsomal membrane [9]. The hydroxylases were more severely affected than the dehydrogenases, although in studies of liver microsomes cytochrome P-450 seemed to be associated with the inner surface. Moreover, our studies of isotope distribution had indicated that intramicrosomal substrate concentration, rather than that of the surrounding medium, was subject to 17,20-lyase action [4] while the reverse seemed true for the 3β-ol-dehydrogenase [10]. The hydroxylases, however, are mixed function oxidases which involve NADPH-cytochrome P-450 reductase (a flavoprotein) and a second factor, probably a phospholipid, to transfer electrons from NADPH to cytochrome P-450. The reductase may well be exposed on the outer surface of the testicular microsomes as it seems to be in the liver microsomes [11], and destruction or solubilization of the NADPH-cytochrome P-450 reductase would interfere with hydroxylations even though the actual interaction of substrate and activating cytochrome P-450 took place within the membrane. The cytochrome P-450 of the mouse testicular microsomes is not

Table 4. Effect of trypsin on binding of [^{14}C]-pregnenolone (centrifugation)

Tube	Microsomes 74 mg testis	Trypsin	Trypsin Inhibition	Min before Inhibition*	% [^{14}C]-Pregnenolone Micr.	Super
A	+	–	–	–	66·5	33·5
B	+	+	+	0	54·8	45·2
C	+	+	+	15	20·1	79·9
D	+	+	+	30	20·8	79·2
E	–	+	+	0	0·9	99·1

* Incubation was at 0°C.

Table 5. Effect of trypsin on steroidogenic enzymes

	Tryps.	Tryps. Inhib.	Min* before Inhib.	Per cent of control activity† 3βDHase	17αOHase	Lyase	17βDHase
Cont.	–	–	–	100	100	100	100
A	+	+	0	96	100	100	86
B	+	+	15	36	i.s.‡	i.s.	25
C	+	+	30	23	i.s.	i.s.	13

* Trypsin incubation at 0–1°C, 0·2 mg/ml.
† Incubation 15 min at 34°C with cofactors.
‡ i.s. = insignificant activity.

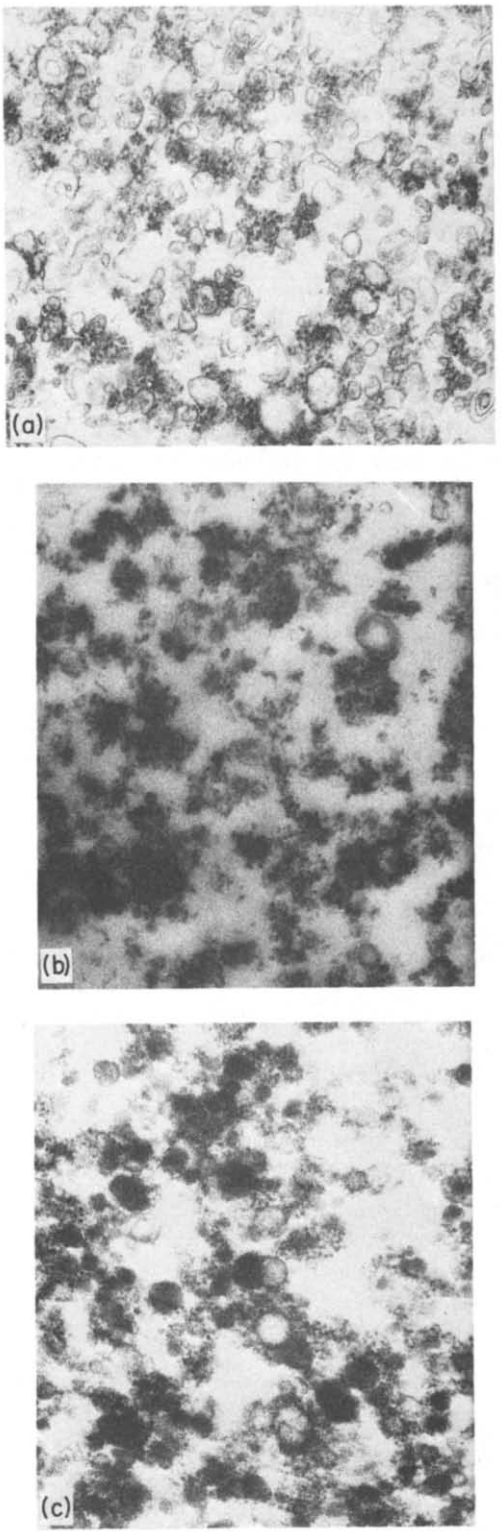

Fig. 1. Electron micrographs of microsomal pellets treated with phospholipases A and C. (a), Control; (b), Phospholipase A; (c) Phospholipase C. Magnification 37,500×.

apparently as firmly bound to the inner membrane, however, as that of the liver cells since it was essentially completely removed by the trypsin treatment used here, while liver microsomal cytochrome P-450 was found by Orrenius *et al.*[11] to be resistant to solubilization by trypsin. Instead, testicular P-450 may be associated with the electron carrier phospholipid in the hydrophobic intermediate region.

It is also not surprising that both phospholipase C and trypsin interfere with differential distribution of pregnenolone. The high capacity-low association constant type of binding could well be due to a lipoprotein or proteolipid which would be destroyed by either enzyme.

The difference between the binding factor, the 3β-hydroxy-dehydrogenase and the trypsin-susceptible unit of the hydroxylating reactions is well illustrated when testis microsomes are treated with 0·049% deoxycholate, a concentration of this detergent sufficient to release some of the proteins which are easily labelled with iodine, and therefore appear to be on the outer surface [12]. As Table 6 shows, under the conditions used there was no significant effect on either binding of 3β-hydroxy-dehydrogenase but the hydroxylation reactions were definitely reduced. Apparently the labile factor is easily displaced from the membrane. This concentration of deoxycholate was sufficient to increase the glucose-6-phosphatase activity.

We can summarize the probable picture at this point as indicating a concentration of both pregnenolone and progesterone in the outer surface of the microsomal vesicle. The 3β-hydroxy-dehydrogenase seems embedded in this outer surface and considerable amounts of pregnenolone are converted to progesterone. The progesterone concentrated in the membrane interacts with a specific site on the cytochrome P-450 which is being maintained in the reduced state by electrons transferred via an intermediate in the membrane, possibly a phospholipid, from NADPH undergoing oxidation at the outer surface. The progesterone then undergoes a hydroxylation type reaction. Let us now examine the evidence regarding the reactions finally leading to testosterone.

Table 7 illustrates the distribution of isotopes in products when various concentrations of [^{3}H]-progesterone and [^{14}C]-17-hydroxyprogesterone are incubated together in the presence of testicular microsomes [13]. As previously reported the conversion of 17-hydroxyprogesterone to androstenedione and testosterone is inhibited by progesterone, apparently competitively. However, the conversion of the 17-hydroxylated intermediate of progesterone to C_{19} steroids is unaffected by the presence of 17-hydroxyprogesterone; the only effect of the latter is inhibition of 17α-hydroxylation.

The strong competition of progesterone for the binding site involved in lyase action indicates that groups common to both compounds react. On the other hand, added 17-hydroxyprogesterone shows no such competition with the intermediate between progesterone and androstenedione but only competes significantly at the 17α-hydroxylase step. These results could best be explained by envisioning progesterone interacting with the cytochrome P-450 complex in

Table 6. Effect of 0·049% deoxycholate (DOC) on microsomal binding and enzyme activity

	Per cent of controls
Pregnenolone binding, centrifuge	100
Progesterone binding, centrifuge	91
3β-Dehydrogenase	94
17β-Hydroxylase	64
17,20-Lyase	66

Table 7. Metabolism of [^{3}H]-progesterone and [^{14}C]-17-hydroxyprogesterone (all values given are μmol/l)

Substrate Prog-[^{3}H]	0	0	0	5	5	5	10	10	10
Substrate 17OHP-[^{14}C]	2·5	5	10	2·5	5	10	2·5	5	10
Compounds recovered									
17,20α-Diol-[^{3}H]				0·31	0·33	0·28	0·60	0·58	0·53
17,20α-Diol-[^{14}C]	0·13	0·28	0·47	0·15	0·31	0·49	0·14	0·29	0·48
17OHP-[^{3}H]				1·12	0·91	0·77	1·50	1·30	0·98
17OHP-[^{14}C]	1·82	3·94	8·48	2·12	4·30	8·86	2·23	4·48	8·78
Testos(T)-[^{3}H]				0·17	0·17	0·11	0·26	0·22	0·19
Testos(T)-[^{14}C]	0·20	0·35	0·43	0·09	0·16	0·24	0·055	0·09	0·17
Δ^4-And(A)-[^{3}H]				0·15	0·13	0·16	0·22	0·20	0·18
Δ^4-And(A)-[^{14}C]	0·24	0·28	0·41	0·05	0·10	0·17	0·04	0·08	0·15
20α-ol-[^{3}H]				0·09	0·09	0·07	0·14	0·13	0·14
Prog-[^{3}H] + Pnane-[^{3}H]				2·92	3·05	3·16	6·90	7·06	7·62
Total T + A-[^{3}H]				0·32	0·30	0·27	0·48	0·42	0·37
Total T + A-[^{14}C]	0·44	0·63	0·84	0·14	0·26	0·41	0·095	0·17	0·32
Total [^{3}H] Compounds*				4·76	4·68	4·55	9·62	9·49	9·64
Total [^{14}C] Compounds*	2·39	4·85	9·79	2·41	4·87	9·76	2·47	4·94	9·58
Total 17OHase-[^{3}H]				1·75	1·54	1·32	2·58	2·30	1·88
Total Lyase-[^{3}H]				0·32	0·30	0·27	0·48	0·42	0·35
[^{3}H]-17OHase/Lyase				5·47	5·13	4·89	5·38	5·48	5·37

* Total recovered in listed compounds.
Incubation 15 min at 34°C in air with NADPH generating system.
All values are averages of duplicate incubations.

such a way that it is first oriented with a group on the enzyme which activates the 17α-hydrogen. The intermediate radical then becomes oriented toward an activating group which carries out the lyase reaction without much dissociation of the intermediate steroid radical from the enzyme complex [14]. There is a small amount of dissociation, however, as indicated by the slow accumulation of 17-hydroxyprogesterone having the progesterone isotope even when large amounts of free 17-hydroxyprogesterone are present.

The conversion of androstenedione to testosterone seems to occur in the same outer region of the microsomal membrane as the 3β-ol-dehydrogenase since various types of treatment seem to affect both dehydrogenases similarly. Androstenedione, testosterone and 17-hydroxyprogesterone appear to diffuse freely in and out of the microsomal vesicle, approaching the distribution of ^3H_2O [5]. Since free 17-hydroxyprogesterone is normally not an intermediate, however, relatively little will leave the cell. As androstenedione diffuses it will interact with 17β-dehydrogenase to a considerable extent, depending on the concentration of the enzyme, and the major compounds diffusing from the active interstitial cells as secretory products will be androstenedione and testosterone. This is our present interpretation of the organization of the enzymes of steroid biosynthesis from pregnenolone to testosterone in the endoplasmic reticulum.

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