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3β-Hydroxy Steroid Dehydrogenase Activity in the Mitochondria of Rat Adrenal Homogenates

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The activity of 3β-hydroxy steroid dehydrogenase (EC 1.1.1.51) in the mitochondrial fraction of rat adrenal homogenates was approx. 31% of the total activity recovered after differential centrifugation and washing of the particulate fractions. Some 45% of the total activity was found in the microsomal fraction. The activity was assayed by a radioisotopic method devised in this laboratory for the purpose of studying small quantities of tissue and cell fractions. Satisfactory separation of the two fractions was demonstrated by electron microscopy of the pellets and by comparative recoveries of RNA, steroid 21-hydroxylase and cytochrome *c* oxidase in the various compartments. Analyses of the kinetics of the enzyme activity in the two fractions revealed no significant differences in apparent K_m for pregnenolone, dehydroepiandrosterone or NAD^+, but demonstrated a distinct difference in the K_m for $NADP^+$. pH optima and susceptibility to cyanoketone inhibition were similar in both fractions.

The sequence of biosynthetic reactions required to produce circulating corticosteroids from cholesterol in the adrenal cortex appears to involve movement of the substrate from one intracellular compartment to another. The initial and terminal steps in the sequence occur in mitochondria. Several of the intermediate reactions are catalysed by enzymes found in the microsomal fraction of adrenal homogenates (Samuels & Uchikawa, 1967). One critical step in the pathway involves the conversion of pregnenolone, which is formed in mitochondria (Koritz, 1968), into progesterone via 3β-oxidation and $\Delta^{5,4}$-isomerization. The first, and rate-limiting, reaction is catalysed by 3β-hydroxy steroid dehydrogenase (3-hydroxy steroid–NAD oxidoreductase; EC 1.1.1.51) (Neville & Engel, 1968). Activity of this enzyme was found in the microsomal fraction of ox adrenal homogenates by Beyer & Samuels (1956). Although this microsomal localization has been generally accepted, in recent years evidence has been accumulating that 3β-hydroxy steroid dehydrogenase activity may be associated with either two enzymes or, in other steroidogenic tissues, may be present in two intracellular locations (Koide & Torres, 1965; Jackanicz & Armstrong, 1968; Sulimovici & Boyd, 1969). McCune, Roberts & Young (1970) have reported the presence of 3β-hydroxy steroid dehydrogenase activity in the mitochondrial fraction of a rat adrenal homogenate, but they did not provide any estimate of the proportion of the total cellular activity in this fraction. Also unresolved is the problem of the number of enzymes actually present. In view of these findings and the possible relationship of 3β-hydroxy steroid dehydrogenase activity to the movement of the steroid molecules, we decided to re-examine the intracellular localization of the activity.

MATERIALS AND METHODS

Adrenal glands of adult Sprague–Dawley rats of either sex were obtained promptly after decapitation, dissected from fat, weighed, quartered and chilled. Homogenates (10g wet wt. of tissue/100ml of buffer) in ice-cold 0.25M-sucrose were prepared in an all-glass Potter-type homogenizer with ten strokes of a loose-fitting pestle. Fractions were made in the cold by the method of Wilson, Nelson & Harding (1965). Each particulate fraction was washed twice and the supernatant fluid discarded after each washing. The efficacy of the separation was evaluated by electron microscopy of mitochondrial and microsomal pellets embedded in agar and by assays of each fraction for cytochrome *c* oxidase (Cooperstein & Lazarow, 1951) as a mitochondrial marker enzyme and for RNA (Schneider, 1957) and steroid 21-hydroxylase (Rosenthal & Narasimhulu, 1969) as indicators of the microsomal fraction.

3β-Hydroxy steroid dehydrogenase was determined by measuring the rate of conversion of either dehydro[^{14}C]-epiandrosterone into androst-4-enedione or [^{14}C]pregnenolone into progesterone. The isomerase reaction is rapid

(Neville & Engel, 1968), so it is customary to refer to the assay in terms of the rate-limiting dehydrogenation step. The reaction mixture contained either 0.478μg of dehydro[^{14}C]epiandrosterone (sp. radioactivity 197μCi/mg) or 0.57μg of [^{14}C]pregnenolone (sp. radioactivity 175μCi/mg) (Amersham–Searle Corp., Arlington Heights, Ill., U.S.A.). The appropriate steroid dissolved in benzene was added to each reaction vessel and evaporated to dryness under a stream of N_2. The radioactive steroids were then dissolved in 3.0μl of propylene glycol and to this solution was added a reaction mixture consisting of 0.23μmol of NAD^+, 1.5mg of bovine serum albumin, 4.0μmol of NaH_2PO_4, 1.0μmol of Na_2HPO_4, and 7.5μmol of NaCl, in a total volume of 0.14ml. The pH was 7.35. To this was added up to 10μl of the putative enzyme solution, containing 0.5–10μg of protein depending on the activity of the preparation. The reaction mixture was prewarmed to 37°C and the reaction started by the addition of the enzyme. It was stopped after 10min by the addition of 100μl of acetone containing 50μg of unlabelled substrate and 50μg of unlabelled product, to which had been added a known quantity of tritiated product (0.05μCi).

After the acetone, 3.0ml of methylene chloride was added to each assay tube and the mixture vigorously agitated for 30s. The upper aqueous phase was removed and replaced with 1.0ml of 0.1M-NaOH. The mixture was again shaken and the aqueous phase removed. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness in a small conical centrifuge tube. Each sample was then redissolved in 30μl of benzene and applied to a silica-gel t.l.c. plate (silica gel F-254; E. Merck A.-G., Darmstadt, Germany). When dehydroepiandrosterone was used as a substrate the chromatograms were developed with chloroform–acetone (4:1, v/v), and when pregnenolone was used the plates were developed in chloroform–ethanol (9:1, v/v). After the plates had been dried, the u.v.-absorbing androst-4-enedione or progesterone region was cut out of the chromatogram, eluted with ethanol and counted for radioactivity in a liquid-scintillation spectrometer (Packard model 3380) by using a scintillator containing 5g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l of toluene. Narrow windows were used to discriminate ^{14}C from ^{3}H and appropriate internal standards were used to determine the efficiency of counting for each radioisotope. Thus ^{14}C was counted at 52% efficiency without significant ^{3}H contamination and ^{3}H was counted at 5.5% efficiency in a channel in which ^{14}C was counted at 1.25% efficiency. The recovery of the added ^{3}H-labelled product was determined and by using this value the quantity of ^{14}C-labelled substrate oxidized was calculated. The identity of the products was further characterized by sequential rechromatography in chloroform–diethyl ether (4:1, v/v) and cyclohexane–ethyl acetate (1:1, v/v) for androstenedione and in benzene–ethanol (9:1, v/v) and cyclohexane–ethyl acetate (1:1, v/v) for progesterone (Lisboa, 1969). At each step of the chromatography authentic unlabelled carrier was added to make possible the visual detection of the product under u.v. illumination. The results of such determinations are shown in Table 1. The effect of several alterations in the components of the reaction mixture are shown in Table 2. Similar results were obtained when pregnenolone was used as a substrate.

The amount of product formed was proportional to the quantity of adrenal protein added over a range of 0.25–10μg when normal adult female rat adrenal tissue was used as the source of the enzyme (Table 2). Bovine serum albumin (or EDTA, disodium salt) was required to stabilize the 3β-hydroxy steroid dehydrogenase activity at low concentrations of enzyme. In its absence the assay method could not be used when less than 5.0μg of adrenal protein was added. The concentration of bovine serum albumin used was not inhibitory and in fact a fivefold increase in bovine serum albumin concentration did not inhibit the reaction (Table 2). The amount of product formed was also directly proportional to the time of incubation for at least 20min under the usual conditions. Less than 5% of the pregnenolone or of the dehydroepiandrosterone was converted into metabolites other than the expected progesterone and androstenedione,

Table 1. *Recovery of substrate and product of the 3β-hydroxy steroid dehydrogenase reaction after sequential t.l.c.*

For experimental details see the text.

Substrate	Product added at end of reactions	T.l.c. system	Radioactivity recovered from u.v.-absorbing spot (d.p.m.) ^{3}H	^{14}C	^{3}H/^{14}C ratio
[^{14}C]Pregnenolone	[^{3}H]Progesterone	Chloroform–ethanol (9:1, v/v)	737000	57800	12.7
		Benzene–ethanol (9:1, v/v)	336200	27300	13.4
		Cyclohexane–ethyl acetate (1:1, v/v)	114500	9210	12.5
Dehydro[^{14}C]epiandrosterone	[^{3}H]Androstenedione	Chloroform–acetone (4:1, v/v)	1105000	29870	37.2
		Chloroform–ether (4:1, v/v)	423000	11350	37.2
		Cyclohexane–ethyl acetate (1:1, v/v)	102300	2723	37.6

Table 2. *Effects of changes in reaction mixture and reaction time on the rate of synthesis of androstenedione by rat adrenal gland 3β-hydroxy steroid dehydrogenase*

The reaction was started by adding portions of a 1% homogenate (2.6μg portion/μl) of rat adrenal gland in 0.25M-sucrose to a dehydroepiandrosterone-containing reaction mixture prewarmed to 37°C. The reaction mixture was prepared as described in the text. The results are the means of duplicate determinations.

	Volume of enzyme (μl)	Time of incubation (min)	Product formed (pmol/min)
Complete mixture	5	10	14.5
	3	10	8.5
	1	10	3.3
	5	0*	0.3
	5	2	14.3
	5	5	14.9
	5	20	14.8
EDTA (disodium salt) (instead of albumin)	5	10	12.9
Albumin omitted	5	10	4.3
NAD^+ omitted	5	10	0.5
5× Bovine serum albumin (7.5mg/tube)	5	10	15.1
5× Dehydroepiandrosterone	5	10	9.5

* Acetone (100μl) added before enzyme sample.

even if the reaction was permitted to continue until 30–40% of the substrate had been converted. These other products were disregarded in the calculations. Under the usual conditions at least 90% of the substrate could be recovered unchanged at the conclusion of the assay. Increasing the concentration of dehydroepiandrosterone twofold was without effect on the activity measured, but a fivefold increase resulted in significant inhibition of enzyme activity (Table 2). Enzyme activity was calculated on the basis of total protein in the homogenates, as measured by the procedure of Lowry, Rosebrough, Farr & Randall (1951). Endogenous steroids or nucleotide cofactors were disregarded in calculations of K_m.

RESULTS

The distribution of 3β-hydroxy steroid dehydrogenase in tissue fractions of a 10% (w/v) homogenate of adult rat adrenal gland is shown in Table 3 and in Fig. 1. In ten such fractionations the recovery of activity averaged 55.33±12.51%. This relatively low yield is attributable to at least two factors. First, yield was sacrificed for purity in that washings were discarded, and, secondly, the stability of the enzyme in isolated tissue fractions is markedly decreased. Some 31% of total activity recovered was present in the mitochondrial fraction, compared with 45% in the microsomal fraction. The ratio of activities measured by using dehydroepiandrosterone or pregnenolone as substrate was constant in all fractions (Table 3). The specific activity of the enzyme in the microsomal fraction was approximately twice that in the mitochondrial fraction. Comparison of the activities of the enzyme in the two fractions revealed a similar apparent K_m for pregnenolone, dehydroepiandrosterone and NAD^+ (Table 4), as determined by double-reciprocal plots of velocity of the reaction versus substrate concentration (Lineweaver & Burk, 1934). Identical pH optima of 7.3 in 0.03M-sodium phosphate buffer and 9.5 in 0.03M-sodium glycine buffer were found (Table 4). The enzyme from both sources was inhibited by cyanoketone (2α-cyano-4,4,17-trimethylandrost-5-ene-17β-ol-3-one, Sterling-Winthrop, Rensselaer, N.Y., U.S.A.) (Table 5). The only difference found was when $NADP^+$ was tested as cofactor. The apparent K_m for $NADP^+$ in the mitochondrial fraction (0.13mM) was an order of magnitude smaller than that for the same cofactor in the microsomal fraction (1.5mM) (Fig. 2).

The results of the fractionation procedure and its efficacy in separating mitochondrial and microsomal fractions are demonstrated in Fig. 1, in which are shown the comparative distributions of cytochrome *c* oxidase, RNA and steroid 21-hydroxylase in the fractions of adrenal homogenates. Some 83% of the cytochrome *c* oxidase activity recovered was found in the mitochondrial fraction, whereas only 13% of the RNA and 10% of the steroid 21-hydroxylase activity were in this fraction. Since over 30% of the 3β-hydroxy steroid dehydrogenase activity was found in this fraction, it is obvious that two to three times as much of this enzyme is present in the mitochondrial fraction as could be accounted for on the basis of fortuitous microsomal contamination of the pellet. RNA was also abundant in the supernatant fraction of these adrenal homogenates, presumably owing to the presence of non-membrane-associated species of RNA. The paucity of 3β-hydroxy

Table 3. *Distribution of 3β-hydroxy steroid dehydrogenase activity in tissue fractions of rat adrenal homogenates*

Both the total activity and that measured in each fraction are given as means±S.D. The pregnenolone-to-progesterone activity was measured in four experiments; the dehydroepiandrosterone-to-androsterone activity was measured in the same four samples plus three additional experiments. The percentage of recovered activity was calculated by dividing the activities found in each of the subcellular fractions by the sum of the activities measured in all of these fractions. Abbreviations: N, nuclear fraction; DM, dense mitochondrial fraction; LM, light mitochondrial fraction; Mic, microsomal fraction; Sup, microsomal supernatant fluid; WH, whole homogenate.

		Tissue fraction						Recovery
Substrate		WH	N	DM	LM	Mic	Sup	(%)
Pregnenolone	nmol of progesterone formed/min per g of tissue	1270±139	30±3.3	242±20	70±17	345±43	63±1.8	58.5
	% of recovered activity	—	4	32	9.5	46	8.5	
Dehydroepiandrosterone	nmol of androstenedione formed/min per g of tissue	938±165	22±10	158±37	51±20	224±56	57±9	54.6
	% of recovered activity	—	4	31	10	44	11	

Table 4. *Properties of 3β-hydroxy steroid dehydrogenase activity in the mitochondrial and microsomal fractions of rat adrenal gland*

The apparent Michaelis constants were determined by using the conditions described in text and in the legend to Fig. 3. pH optima were determined by replacing the usual phosphate buffer with the appropriate sodium phosphate or sodium glycine buffer. The final concentration of the buffer in the reaction mixture was 0.033 M.

	K_m (pregnenolone) (M)	K_m (dehydroepiandrosterone) (M)	K_m (NAD^+) (M)	K_m ($NADP^+$) (M)	pH optimum	
					Phosphate buffer	Glycine buffer
Mitochondrial fraction	1.7×10^{-6}	0.35×10^{-6}	0.029×10^{-3}	0.13×10^{-3}	7.3	9.5
Microsomal fraction	0.81×10^{-6}	0.46×10^{-6}	0.031×10^{-3}	1.5×10^{-3}	7.3	9.5

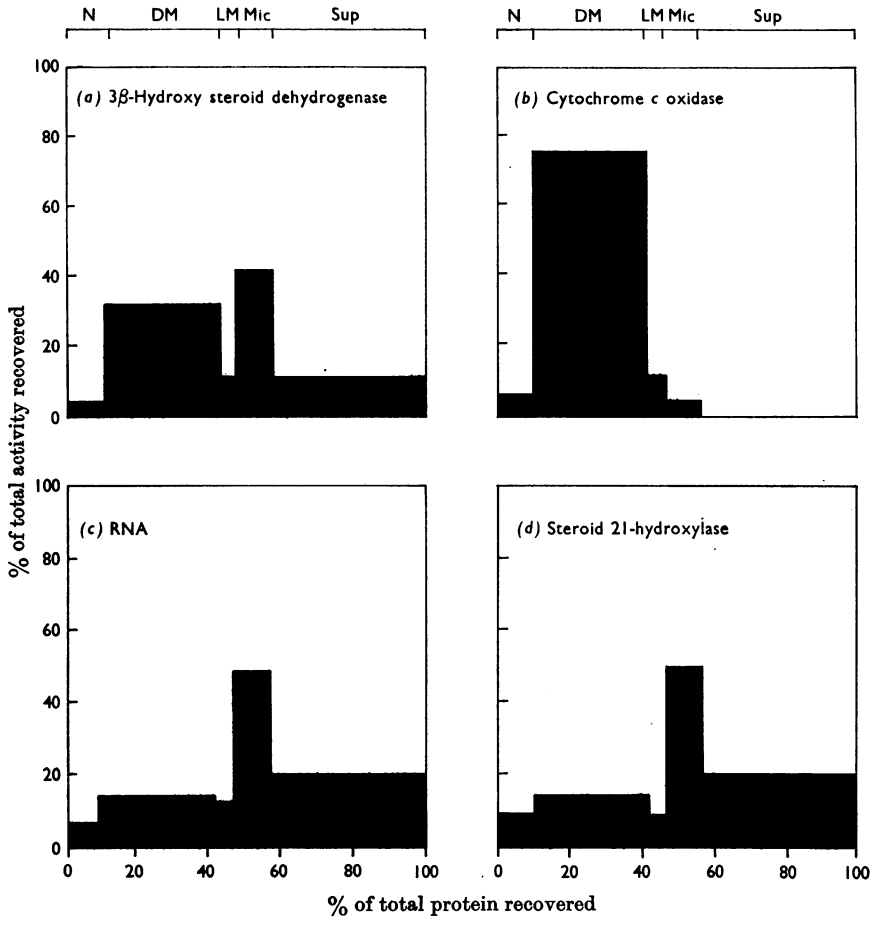

Fig. 1. Distribution pattern of 3β-hydroxy steroid dehydrogenase, cytochrome *c* oxidase, steroid 21-hydroxylase and RNA in subcellular fractions of rat adrenal gland. The fractions were prepared and assayed as described in the text. The abbreviations are as in Table 3. '% of total protein recovered' refers to the fraction of the protein present in the original homogenate recovered in each of the subcellular fractions.

steroid dehydrogenase activity in the light-mitochondrial fraction is not a consequence of the destruction of some component of the reaction mixture. When mixtures of the dense-mitochondrial plus light-mitochondrial fractions or light-mitochondrial plus microsomal fractions were assayed, the measured activity was the sum of the activities found in each component of the mixture. Examination of the mitochondrial pellet in the electron microscope revealed relatively good preservation of the mitochondria, although there was an increase in matrix density and some loss of cristae. Relatively few extramitochondrial vesicles were present, and these could not be distinguished from portions of cristae derived from ruptured mitochondria or Golgi vesicles. No mitochondria were seen in the microsomal pellet, which consisted almost exclusively of smooth vesicles.

DISCUSSION

Although the activity of 3β-hydroxy steroid dehydrogenase in rat adrenal homogenates and slices had been studied (Rubin, Deane & Hamilton, 1963; Schor & Glick, 1967), the subcellular localization of the activity in this species has been examined only by Inano, Machino & Tamaoki (1969) and by McCune *et al.* (1970). Inano *et al.* (1969) limited their study to the distribution of activity within the microsomal compartment and they found most of it in the ribosome-free fraction.

Table 5. *Inhibition by cyanoketone of 3β-hydroxy steroid dehydrogenase activities in mitochondrial and microsomal fractions of rat adrenal gland*

The reaction mixture containing [^{14}C]pregnenolone was preincubated with cyanoketone for 30 min at 37°C before the enzyme was added (enzyme solution: 10 μg of mitochondrial protein, 2.2 μg of microsomal protein).

Concn. of cyanoketone (μM)	Progesterone synthesized (pmol/min)	
	Mitochondrial enzyme	Microsomal enzyme
0	6.50	6.30
1.00	—	0.007
0.50	0.007	0.005
0.30	0.30	0.30
0.20	0.67	0.67
0.10	1.40	1.20
0.067	1.95	1.40
0.033	4.00	2.60
0.01	6.20	6.20

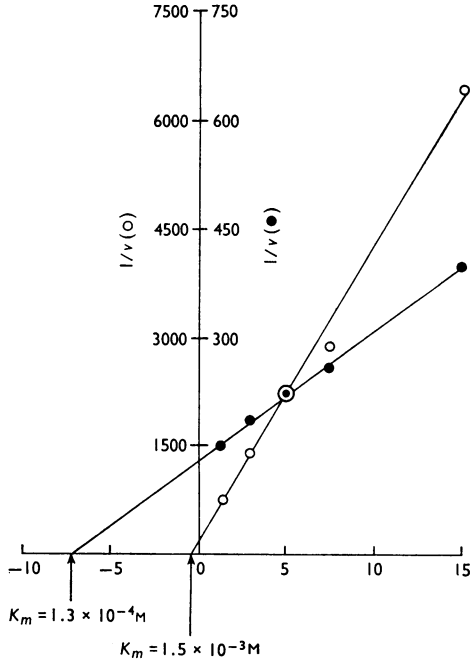

Fig. 2. Determination of the apparent Michaelis constant (K_m) for NADP$^+$ of mitochondrial (●) and microsomal (○) 3β-hydroxy steroid dehydrogenases from rat adrenal gland. The conditions were essentially those described in the Materials and Methods section. The velocity of the reaction is given in terms of nmol of androstenedione produced from dehydroepiandrosterone. The mitochondrial enzyme was 5.0 μg of mitochondrial protein and the microsomal enzyme was 2.1 μg of microsomal protein.

McCune *et al.* (1970) evaluated the inhibition of 3β-hydroxy steroid dehydrogenase activity by cyclic AMP in the mitochondrial and microsomal fractions of a rat adrenal homogenate, but did not report the distribution of activity in a complete fractionation. Our findings indicate that a substantial proportion of 3β-hydroxy steroid dehydrogenase activity in the rat adrenal is found in the mitochondrial fraction.

Similar observations have been made in rat ovarian tissue by Sulimovici & Boyd (1969) and in human placental tissue by Koide & Torres (1965). Our results indicate that it is not an artifact of the fractionation technique. The distribution of the marker enzymes studied indicates that far more of the 3β-hydroxy steroid dehydrogenase activity is present in the mitochondrial fraction than could be explained on the basis of fortuitous contamination with microsomal components. Like Sulimovici & Boyd (1969), we find that the properties of the enzymes assayed in the two fractions are similar with regard to apparent K_m for pregnenolone and NAD$^+$ and in pH optima, and differ with regard to NADP$^+$ as a cofactor. As yet we cannot conclusively distinguish between the possibilities that the slight differences noted in the properties of the enzymes reflect differences in the structure of two different enzymes, as suggested by Koide & Torres (1965), or are a consequence of the association of a single enzyme with different organelles.

The possible significance of this observation for our understanding of steroid biosynthesis remains to be explored. Close approximation of smooth endoplasmic reticulum to mitochondria in cells of the zona fasciculata has been described in electron-microscopic studies of the adrenal cortex after a variety of physiological stimuli of steroid synthesis (Lever, 1956; Nishikawa, Murone & Sato, 1963; Borowicz, 1965; Luse, 1967). We may be measuring a biochemical consequence of this relationship, as the microsomal fraction obtained from the adrenal homogenate is composed almost entirely of smooth endoplasmic reticulum (Sabatini & De Robertis, 1961). A physiological union of mito-

chondria and some portion of the endoplasmic reticulum would eliminate the apparent necessity for the transport of pregnenolone out of mitochondria. If such is the case, the difference in distribution of 3β-hydroxy steroid dehydrogenase and steroid 21-hydroxylase activities may be evidence that the smooth endoplasmic reticulum of the adrenal cortex, like that of the liver (Dallner & Ernster, 1968), is a functionally heterogeneous organelle.

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