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LOCALIZATION OF 3-β-HYDROXYSTEROID DEHYDROGENASE IN THE INNER MEMBRANE SUBFRACTION OF RAT TESTIS MITOCHONDRIA

SOREL SULIMOVICI, BENJAMIN BARTOOV AND BRUNO LUNENFELD

Institute of Endocrinology, The Chaim Sheba Medical Center, Tel Hashomer and the Department of Life Sciences, Bar-Ilan University (Israel)

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SUMMARY

1. The activity of 3-β-hydroxysteroid dehydrogenase (3-β-hydroxysteroid: NAD (P) oxidoreductase, EC 1.1.1.51) in rat testis mitochondria and submitochondrial fractions was determined.

2. Inner and outer mitochondrial membranes were obtained by subjecting the mitochondrial pellets to controlled osmotic lysis, ultrasonic vibration, and treatment with a non-ionic detergent, Lubrol WX, followed by differential centrifugation.

3. The purity of the isolated mitochondria and of mitochondrial membranes was ascertained by electron microscopy, measurement of enzyme markers and by a method based upon labelling of the mitochondrial and cytoplasmic RNA with [5-^{3}H]orotic acid.

4. The results of this study suggest that the 3-β-hydroxysteroid dehydrogenase is present in rat testis mitochondria, free of endoplasmic reticulum and that this enzyme is mainly localized in the inner mitochondrial membrane.

INTRODUCTION

Steroidogenic tissues contain an enzyme system which catalyzes the oxidation of the Δ^5-3β-hydroxy group of both C_{21} and C_{19} steroids to form the Δ^4-3-oxogroup[1]. It has been established that this reaction requires the participation of a 3-β-hydroxysteroid dehydrogenase (3-β-hydroxysteroid:NAD (P) oxidoreductase, EC 1.1.1.51) and a Δ^5-3-oxosteroid isomerase (3-oxosteroid Δ^4-Δ^5-isomerase, EC 5.3.3.1)[2–4]. These enzymes have been localized in the microsomal fraction of the adrenal cells[5] and subsequent subfractionation of adrenal microsomes demonstrated their presence in smooth surfaced microsomes[6] which are ribosome-free membranes. Although the microsomal localization has been generally accepted, in the last few years evidence has been accumulated that these enzymes might also be attached to the mitochondrial fraction of different steroidogenic tissues. A bimodal distribution of a dehydrogenase–isomerase system has been reported in mitochondria and microsomal fractions isolated from human term placenta[7], ovarian tissue[8,9] and adrenal cor-

tex[10,11]. However, since these enzymes have been associated with the microsomal fraction[5,6] their presence in mitochondria could be related to microsomal contamination.

The present experiments were designed to investigate whether the 3-β-hydroxysteroid dehydrogenase is present in a pure mitochondrial preparation, and if so, to separate the mitochondrial membranes in order to establish the submitochondrial localization of this enzyme. Mitochondria isolated from rat testis served as the enzyme source. Portions of these studies have appeared in an abstract form[12].

MATERIALS AND METHODS

Source of materials

[7α-^{3}H]Progesterone (spec. act. 8.5 Ci/mmole) and [5-^{3}H]orotic acid (spec. act. 18 Ci/mmole) were obtained from the Radiochemical Centre, (Amersham, England). The radiochemical purity of progesterone was verified by thin-layer chromatography on silica gel-G using benzene–ethyl acetate (4:1, by vol.) together with standard reference samples. Unlabelled pregnenolone and progesterone were purchased from Ikapharm (Israel) and were assayed for purity by thin-layer and gas–liquid chromatography. Nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$), bovine serum albumin and cytochrome *c* (horse) were obtained from Sigma (U.S.A.). The nonionic detergent Lubrol WX was from I.C.I. (England). Other chemicals and organic solvents were of analytical reagent grade. The organic solvents were purified by simple distillation.

Preparation of mitochondria

Testicular tissue was obtained from mature male rats (Charles River Colony) 2–3 months old weighing 250–300 g. The rats were killed by a sharp blow on the head. The testes were removed, decapsulated, weighed and kept on ice until the next stage of preparation. Within 10 min of killing the animals the testicular tissue was homogenized in 5 vol. of ice-cold 0.25 M sucrose with a homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 600 $\times$ *g* for 10 min. The supernatant fluid was carefully decanted and retained while the sediment was washed with 1 vol. 0.25 M sucrose and again centrifuged at the same speed for a further 10 min. The combined supernatant fluids were centrifuged at 9000 $\times$ *g* for 20 min to sediment the mitochondria which were resuspended in 0.154 M KCl by hand homogenization and centrifuged at 9000 $\times$ *g* for 10 min. This procedure was repeated 5 times.

Preparation of submitochondrial fractions

The washed mitochondrial pellet was suspended in Parson's swelling medium[13] consisting of 20 mM phosphate buffer, pH 7.2, to which bovine serum albumin was added to a final concentration of 0.02%. This suspension was incubated at 4 °C for 20 min and then centrifuged at 34 000 $\times$ *g* for 20 min to obtain a pellet of lysed mitochondria. These were suspended by hand homogenization in 2 vol. of distilled water and subjected to ultrasonication at 0 °C for 3 min (with intervals for cooling every minute) using a Bronson B12 sonifier cell disrupter, operating at 20 kcycles/s. The sonicated mitochondria were treated with non-ionic detergent Lubrol WX at a concentration of 0.05 mg/mg mitochondrial protein and incubated at 4 °C for 20 min.

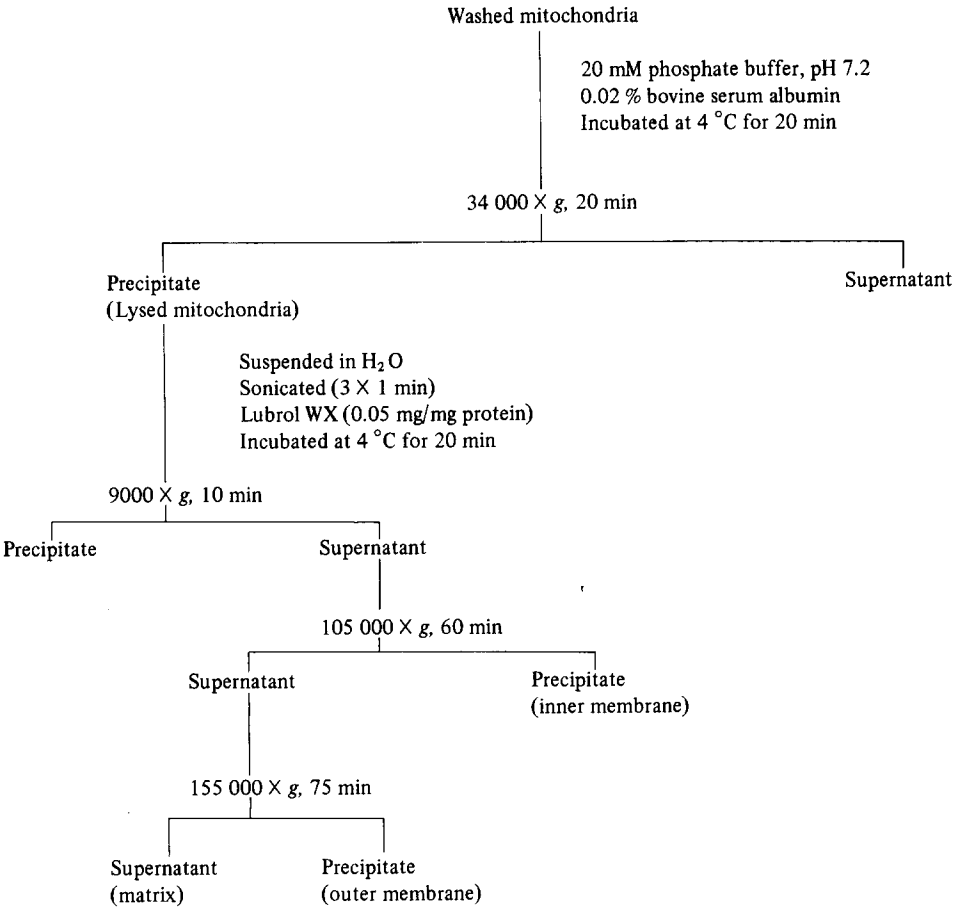

Fig. 1. Diagram outlining the procedure used to fractionate the washed mitochondria.

The mitochondria were then fractionated by differential centrifugation[14]. The unbroken mitochondria were sedimented following centrifugation at 9000 × *g* for 10 min. The 9000 × *g* supernatant fluid was collected and centrifuged at 105 000 × *g* for 60 min, to sediment the inner membrane subfraction. The 105 000 × *g* supernatant was further centrifuged at 155 000 × *g* for 75 min to isolate the outer membrane subfraction. The outline of the procedure is illustrated in Fig. 1.

Evaluation of mitochondrial contamination with endoplasmic reticulum

The purity of the mitochondria and of the isolated mitochondrial membranes was evaluated by labelling cytoplasmic and mitochondrial RNA with [5-^{3}H]orotic acid[15,16]. Two rats were injected intraperitonaly with [5-^{3}H]orotic acid (9 Ci/mmole) 48 h before sacrifice. The untreated animals were kept under identical conditions. The animals were killed and the testicular tissue collected, homogenized and fractioned separately as described before. The unlabelled mitochondria isolated from the untreated animals were cross-contaminated by resuspension in the radioactive 9000 × *g* supernatant fluid isolated from [5-^{3}H]orotic acid-injected animals. This

reconstituted homogenate was stored for 10 min, to promote equilibrium between the labelled and unlabelled fractions. The determination of radioactivity of mitochondria and of different fractions was carried out as follows: 0.1 ml of each sample was mixed with 0.1 ml of a 18% (w/v) Lubrol WX solution. The mixture was placed in a counting vial to which 15 ml of scintillation fluid were added. The counting mixture was composed of 1,4-dioxane:2, ethoxyethanol:xylene: (3:3:1, by vol.) in which 8% naphthalene, 1% 2,5-diphenyloxazole (PPO) and 0.05% 1,4-bis-(-5-phenyloxazolyl-2)-benzene (POPOP) were dissolved. All samples were counted in a Packard Tri-Carb Model 2002 liquid scintillation spectrometer with an efficiency for tritium of 30% and were corrected for quenching.

Enzyme assays

The 3-β-hydroxysteroid dehydrogenase was measured by the conversion of pregnenolone to progesterone. This reaction is catalyzed by a 3-β-hydroxysteroid dehydrogenase and a Δ^5-3-oxosteroid isomerase. Since the latter reaction is rapid[17] the present assay measures the rate-limiting dehydrogenation step[11]. The intact mitochondria, and the mitochondrial subfractions were suspended in 0.1 M phosphate buffer, pH 7.4, and incubated in the presence of magnesium sulphate, NAD^+ and pregnenolone as substrate. The incubations were stopped by adding 0.2 ml of acetic acid followed by freezing at -20 °C. Known amounts of [7α-^{3}H]progesterone were added to the incubation mixture prior to the extraction. The mixture was then extracted three times with ethyl acetate (10 ml). The combined extracts were evaporated to dryness under nitrogen. The dried extract was dissolved in small amounts of chloroform and chromatographed on silica-gel thin-layer plates. The thin-layer plates were run in chloroform–ethyl acetate (8:2, by vol.) together with standard reference samples. This solvent system and technique separated pregnenolone (R_F 0.41) from progesterone (R_F 0.56). The area on the sample lanes corresponding in chromatographic mobility to authentic progesterone was scraped from the plates and eluted with methanol (3 × 4 ml). The samples were dried and the residue dissolved in carbon disulphide and aliquots were taken for analysis by gas–liquid chromatography. An aliquot was also used to measure the recovery of radioactive progesterone. At this stage 85–90% of the added [7α-^{3}H]progesterone was recovered. All the progesterone values reported in this paper have been corrected for losses during extraction and separation with thin-layer chromatography.

A Packard Model 871 gas chromatograph equipped with a hydrogen flame ionization detector was used for quantitation of progesterone. The conditions of operation and the retention time of progesterone in relation to pregnenolone acetate (internal standard) were as previously described[18]. Calibration curves for progesterone and pregnenolone acetate were established and the response of the flame ionization detector was linear for both compounds. A standard curve was prepared by plotting peak area against mass of steroid injected. The amount of progesterone present in the sample injected was determined from the standard curve and corrected for losses during injection. The enzymic activity was expressed as nmoles progesterone formed/min per mg protein. Protein concentration was measured by the method of Lowry *et al.*[19] with bovine serum albumin used as standard.

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) was determined at 37 °C according to the method of Harper[20]. The inorganic phos-

phate released during the incubation was measured after the technique described by Gomori[21]. Succinate dehydrogenase (succinate: (acceptor) NAD oxidoreductase, EC 1.2.99.1) was determined by monitoring spectrophotometrically at 600 nm the succinate dependent reduction of indophenol to leucoindophenol[22]. The extinction coefficient of indophenol was taken to be $16.1 \cdot 10^3$ $cm^{-1} \cdot M^{-1}$.

Cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) was measured spectrophotometrically at 550 nm at room temperature[23]. Horse heart cytochrome *c* was reduced with sodium dithionite. Each preparation was checked for the 550 nm/565 nm absorbance ratio[24] and was only used when this exceeded 6–10.

Glutamate dehydrogenase (L-glutamate:NAD^+ oxidoreductase, EC 1.4.1.2) activity was determined at 25 °C by measuring spectrophotometrically at 340 nm the rate of NAD^+ reduction to NADH[25]. The technique described by Deitrich and Erwin[26] was employed for the determination of monoamino oxidase (monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4). The production of *p*-dimethylaminobenzaldehyde from the corresponding amine was followed spectrophotometrically at 355 nm.

Electron microscopy

The fractions obtained throughout the above mentioned procedure for the isolation of mitochondria and of mitochondrial membranes were negatively stained according to the method of Parson[27]. The surface spreading method was not used; instead collodion coated grids were allowed to float on top of a drop of an unfixed mitochondrial suspension in 0.154 M KCl (5 mg protein/ml) for 1 min. Excess mitochondrial suspension was removed with a filter paper and the grid allowed to float on top of either a 2% (w/v) potassium phosphotungstate solution, pH 6.2 for 2 min or 3.5% (w/v) ammonium molybdate solution pH 7.2 for 2 min[28]. Excess potassium phosphotungstate or ammonium molybdate was removed with a filter paper and the grids were allowed to dry. Specimens were examined in a Jeol Jem 100B electron microscope at 80–100 kV accelerating voltage, and viewed at magnification of 80 000–220 000.

RESULTS

Evaluation of the purity of the mitochondria

In order to evaluate the contamination of mitochondria with microsomes glucose-6-phosphatase, a microsomal marker enzyme[29] was determined. The glucose-6-phosphatase activity of mitochondria before washing (crude) and of washed mitochondria preparations was compared with the glucose-6-phosphatase activity of microsomal fraction prepared from the same tissue (Table I). The specific activity of glucose-6-phosphatase in crude testis mitochondria was reduced from an initial value of 36.88 nmoles/mg protein per min to a value of 1.66 nmoles/mg protein per min after five consecutive washes, each of 0.154 M KCl. On the basis of the comparison of the glucose-6-phosphatase activity in the crude mitochondrial suspension with the corresponding activity in the microsomal fraction an estimate was made of the degree of contamination of mitochondria by microsomes and was found to be 22.06%. The glucose-6-phosphatase contamination of mitochondria washed five successive times was reduced to 1%. In parallel experiments the microsomal fraction was washed with

TABLE I

SPECIFIC ACTIVITY OF GLUCOSE-6-PHOSPHATASE IN RAT TESTIS MITOCHONDRIA PRIOR TO AND AFTER WASHINGS AND IN RAT TESTIS MICROSOMES

Subcellular fraction	*No. of washes*	*Glucose 6-phosphatase* (nmoles/min per mg protein)*	*Microsomal contamination*
Mitochondria	0	36.88 ± 0.43 (5)	22.06
	1	27.12 ± 0.29 (5)	16.22
	2	16.14 ± 0.23 (5)	9.65
	3	8.32 ± 0.19 (5)	4.97
	4	4.00 ± 0.15 (5)	2.39
	5	1.66 ± 0.19 (5)	1.00
Microsomes**	0	167.00 ± 2.00 (6)	–

* Glucose-6-phosphatase activity is expressed as nmoles of Pi released/min per mg protein. Results are expressed as mean ± S.E. of the mean followed by the no. of observations in parenthesis.

** Microsomal fraction was obtained following centrifugation of the 9000 × *g* supernatant fluid of testicular homogenate at 105 000 × *g* for 60 min.

0.154 M KCl in order to establish if the microsomal glucose-6-phosphatase was stable under these conditions. It was found that the specific activity of the enzyme was the same or slightly higher after washing the microsomes four times.

The purity of the mitochondria was further evaluated by labelling of cytoplasmic and mitochondrial RNA with [5-^{3}H]orotic acid. The results illustrated in Table II indicate that the 9000 × *g* supernatant fluid after the third mitochondrial washing of both preparations (mitochondria isolated from the [5-^{3}H]orotic acid-injected animals and mitochondria isolated from the cross-contamination experiments) were devoid of radioactivity. The washed mitochondria isolated from the animals injected with [5-^{3}H]orotic acid retained 0.16% of the tritium whereas the

TABLE II

DISTRIBUTION OF THE RADIOACTIVITY INCORPORATED IN RNA AMONG SUBCELLULAR FRACTIONS ISOLATED FROM RAT TESTIS AFTER INJECTION WITH [5-^{3}H] OROTIC ACID

Group A, rats injected with (5-^{3}H) orotic acid; Group B, non injected rats. The crude testicular mitochondria preparation isolated from Group B was cross contaminated with the 9000 × *g* supernatant fluid isolated from Group A (see arrow). Results are expressed as ^{3}H dpm per subcellular fraction and are given as mean ± S.E. of the mean of three determinations.

Subcellular fraction	*^{3}H (dpm)*	
	Group A	*Group B*
Testicular homogenate	7 000 000 ± 18 000	
9000 × *g* supernatant	6 400 000 ± 10 000	→ 6 400 000 ± 10 000
Crude mitochondria pellets (unwashed)	227 000 ± 4 500	224 000 ± 4 000
First mitochondria wash	200 000 ± 7 000	210 000 ± 3 700
Second	9 000 ± 100	9 400 ± 100
Third	0	0
Fourth	0	0
Fifth	0	0
Washed mitochondria pellets	11 500 ± 500	2 500 ± 70
Inner membrane	3 550 ± 150	1 260 ± 130
Outer membrane	350 ± 50	0

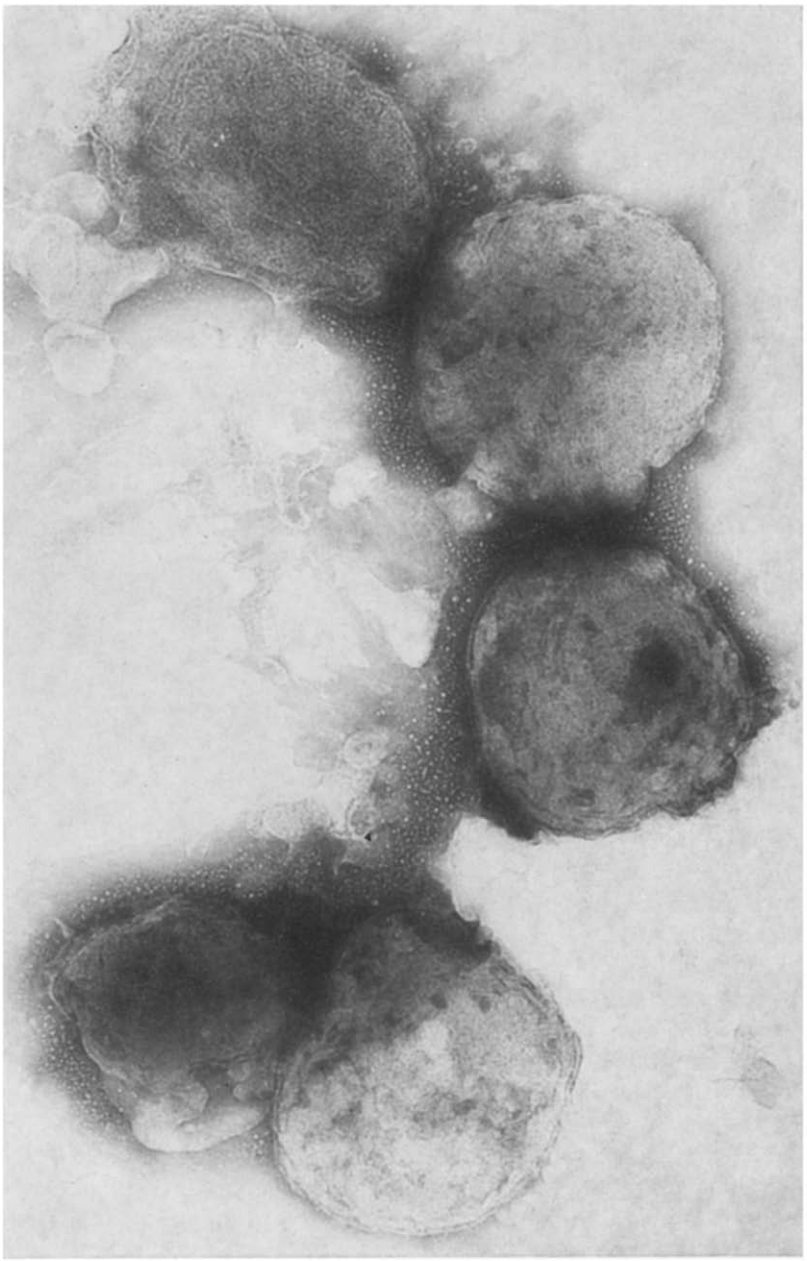

Fig. 2. Electron micrograph of an intact mitochondrial preparation, negatively stained with 3.5% (w/v) ammonium molybdate solution, pH 7.2. Magnification × 60 000, magnification of original micrograph × 80 000.

same preparation isolated from the cross-contamination experiments retained only 0.04% of the total radioactivity added.

Electron microscopy

Fig. 2 shows an electron micrograph of a washed mitochondrial preparation

TABLE III

SPECIFIC ACTIVITY OF SOME MARKER ENZYMES IN INTACT MITOCHONDRIA AND MITOCHONDRIAL MEMBRANES OF RAT TESTIS

Assay mixture: (1) for succinate dehydrogenase, 30 μmoles potassium phosphate (pH 7.4) 0.5 mg bovine serum albumin, 10 μmoles succinate, 20 mg 2,6-dichlorophenol indophenol and 1–2 mg mitochondrial protein to a total volume of 3.5 ml. (2) For cytochrome oxidase, 35 mmoles potassium phosphate buffer (pH 7.4) 56 μmoles reduced cytochrome *c* and 1 mg mitochondrial protein to a total volume of 3 ml. (3) For monoamino-oxidase, 50 mmoles sodium phosphate buffer (pH 7.6) 0.1 ml 0.03% Lubrol WX, 0.3 μmole *p*-dimethylaminobenzylamine and 2–5 mg mitochondrial protein to a final volume of 3 ml. (4) For glutamate dehydrogenase 20 mmoles potassium phosphate (pH 7.7) 30 mmoles nicotinamide, 0.4 mmole KCN, 0.8 mg/ml bovine serum albumin, 13 mmoles potassium glutamate, 1.4 mmoles NAD^+, and mitochondrial protein (2–5 mg) to a total volume of 3 ml.

Enzymes	*nmoles/min per mg protein*		
	Intact mitochondria	*Inner membrane*	*Outer membrane*
Succinate dehydrogenase*	435	515	120
Cytochrome oxidase**	1670	1750	210
Monoamino oxidase***	14.6	2.5	20.8
Glutamate dehydrogenase†	260	370	–

* nmoles of indophenole reduced/min per mg protein.
** nmoles cytochrome *c* oxidized/min per mg protein.
*** nmoles *p*-dimethylaminobenzylamine oxidized/min per mg protein.
† nmoles NAD^+ reduced/min per mg protein.

negatively stained with ammonium molybdate. The majority of the mitochondria were found to be intact, and the difference between the inner and outer membrane is visible. Some of the mitochondria are broken, and there are some fragments of the outer membrane. There was no detectable endoplasmic reticulum contamination. The diameter of these mitochondria was found to be 0.6–0.75 μm.

Characterization of the mitochondrial membranes

Distribution of marker enzymes. The distribution pattern of the outer membrane marker enzyme such as: monoamino-oxidase and the inner membrane marker enzymes *eg.* succinate dehydrogenase, cytochrome oxidase and glutamate dehydrogenase in intact mitochondria and in the submitochondrial fractions is shown in Table III. The monoamino-oxidase was found to be associated mainly with the 155 000 × *g* precipitate whereas cytochrome oxidase, succinate dehydrogenase and glutamate dehydrogenase were mainly with the 105 000 × *g* precipitate. No glutamate dehydrogenase could be detected in the 155 000 × *g* precipitate.

The degree of contamination of the inner and outer mitochondrial membranes with endoplasmic reticulum was assessed by measuring the radioactivity of the submitochondrial fractions isolated from testicular tissue of rats injected with [5-^{3}H]-orotic acid (Table II). It can be seen that the radioactivity in the submitochondrial fractions is very low.

Electron microscopy. Fig. 3 shows a micrograph obtained after staining the 105 000 × *g* precipitate of the mitochondria. It consists of inner membrane fragments of various sizes. Some of them have a circular shape as a result of sonication.

Fig. 4 is a micrograph obtained after staining the 155 000 × *g* precipitate of the mitochondria. It consists of small vesicles, bordered by a single membrane

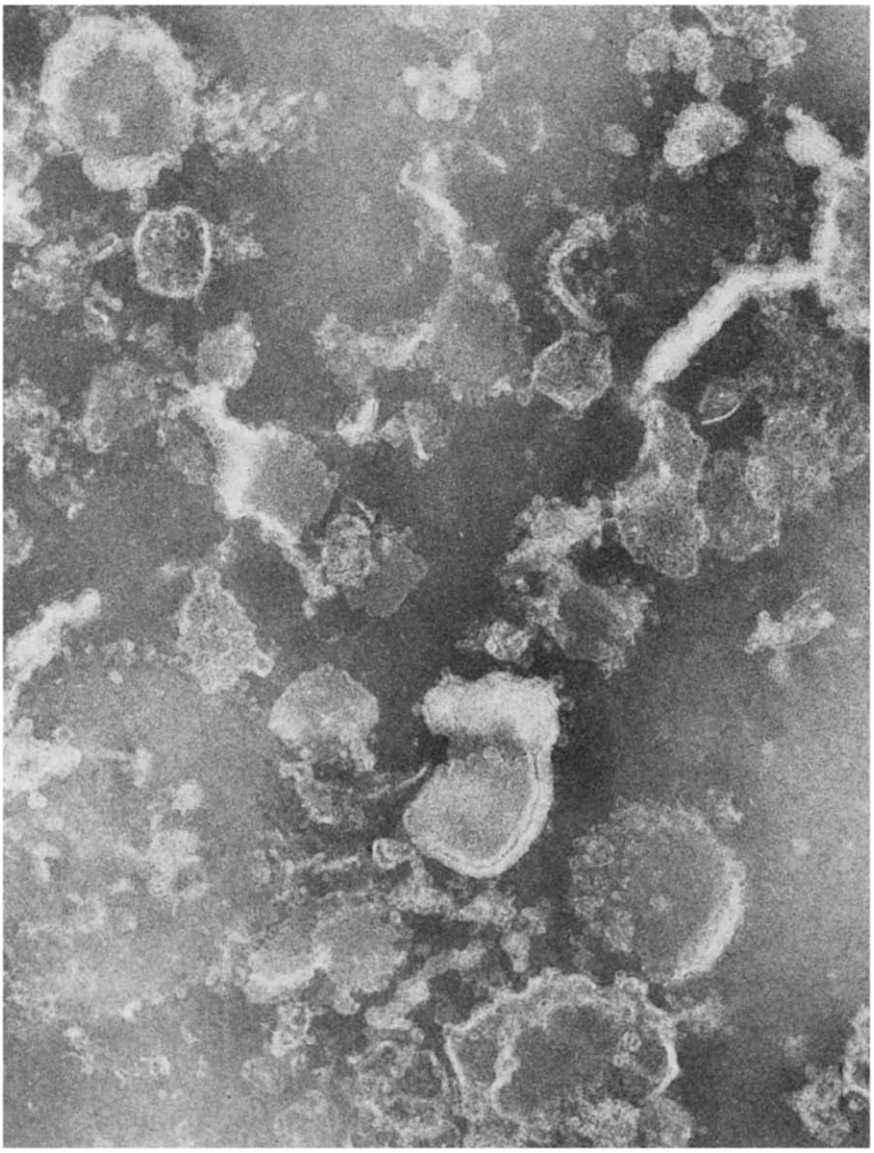

Fig. 3. Electron micrograph of a 105 000 × *g* ppt negatively stained with 2% (w/v) potassium phosphotungstate, pH 6.2. This fraction consists of the inner membrane of mitochondria. Magnification × 165 000, magnification of original micrograph × 220 000.

characteristic of the outer mitochondrial membrane.

In both membranes the endoplasmic reticulum was not visible.

Distribution of 3-β-hydroxysteroid dehydrogenase activity

The conversion of pregnenolone to progesterone by an intact mitochondrial preparation and different submitochondrial fractions is shown in Table IV. Most of the activity was localized in the inner membrane fraction. The outer membrane contained only 1/7 of the enzymic activity. The 155 000 × *g* supernatant (matrix + cell sap) was not capable of converting pregnenolone to progesterone.

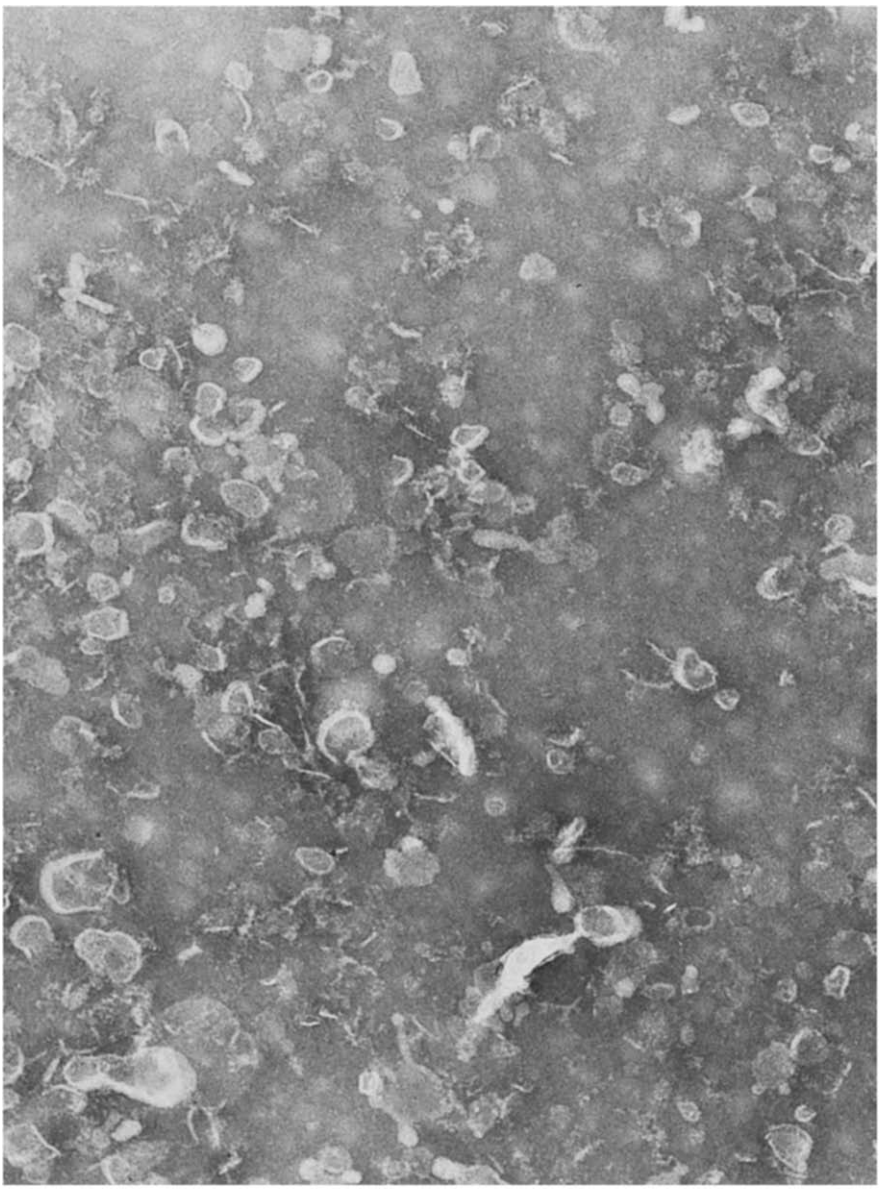

Fig. 4. Electron micrograph of a 155 000 × *g* ppt negatively stained with 2% (w/v) potassium phosphotungstate, pH 6.2. This fraction consists of the outer membrane of mitochondria. Magnification × 135 000, magnification of original micrograph × 180 000.

DISCUSSION

Several investigators have reported some similarity between endoplasmic reticulum and outer mitochondrial membrane[30,31]. This observation raised the problem of the purity of mitochondrial preparations in studies dealing with intracellular localization of enzymes. A diffuse distribution of the enzymes which convert the Δ^5-3β-hydroxysteroids to Δ^4-3-oxosteroids in mitochondria and microsomal fractions of different steroidogenic tissues have been reported[7–11]. One purpose of the present

TABLE IV

DISTRIBUTION OF 3-β-HYDROXYSTEROID DEHYDROGENASE* IN INTACT MITOCHONDRIA AND SUB-MITOCHONDRIAL FRACTIONS OF RAT TESTIS

Incubation mixture: 5 mg protein in 0.1 M phosphate buffer, pH 7.4, 50 μmoles magnesium sulphate, 0.15 μmoles NAD^+ and 0.16 μmole pregnenolone. The total volume was 2 ml and incubations were carried out at 37 °C in air for 20 min.

Subcellular fraction	*Progesterone (nmoles/min per mg protein)*
Intact mitochondria	0.35 ± 0.02 (5)
Inner membrane (105 000 × *g* precipitate)	0.74 ± 0.02 (6)
Outer membrane (155 000 × *g* precipitate)	0.11 ± 0.02 (5)
155 000 × *g* supernatant	0.00

* The 3-β-hydroxysteroid dehydrogenase activity is expressed as nmoles progesterone formed/min per mg. protein and was calculated as described in the section on Methods. Results are given as mean ± SE of the mean followed by the no. of observations in parenthesis.

investigation was to substantiate the accumulated evidence that the presence of 3-β-hydroxysteroid dehydrogenase in a preparation of rat testis mitochondria does not originate from microsomal contamination. The purity of the rat testis mitochondria was assured by biochemical and morphological means. Determination of glucose-6-phosphatase (Table I) demonstrated that the crude mitochondrial preparation contained 22.06% of the phosphatase activity found in microsomal fraction. It should be noted that contamination of mitochondria with this enzyme was reduced after five washes. Thus, repeated washing of mitochondrial preparations significantly reduced the levels of glucose-6-phosphatase. The purity of the mitochondrial fraction was further evaluated by labelling the mitochondrial and cytoplasmic RNA with [5-^{3}H]orotic acid and following the radioactivity of different fractions obtained during the isolation procedure. This was considered to be a reliable method since the RNA content of mitochondria was found to be much lower than that of microsomes. Published values of rat liver mitochondria are of the order of 3 μg RNA per 100 mg protein[16] whereas for rat liver microsomes about 20 mg RNA per 100 mg protein[32,33].

It was observed that in rats injected with [5-^{3}H]orotic acid the majority of the radioactivity was associated with the 9000 × *g* supernatant of testicular homogenates (Table II). This fraction was cross contaminated with a crude mitochondrial preparation isolated from untreated rats. The cross-contamination experiment differentiated between the labelling of mitochondrial and microsomal RNA. The results illustrated in Table II demonstrate that after the third mitochondrial wash, no radioactivity was recovered in the supernatant in both orotic acid-injected animals and in the cross-contamination experiments. This indicated that the bulk of rough endoplasmic reticulum and ribosomes were removed after the mitochondrial preparation was washed three consecutive times with 0.154 M KCl. At this stage 4.97% of the microsomal glucose-6-phosphatase activity was found in mitochondria (Table I). It should be emphasized that this enzyme is located mainly in the smooth endoplasmic reticulum. After two more washes of the mitochondria, the phosphatase contamination was reduced to 1%. This indicates that it is more difficult to remove the smooth endoplasmic reticulum from mitochondria. The washed mitochondrial fraction isolated from rats injected with [5-^{3}H]orotic acid retained 0.16% of the

total radioactivity whereas the washed mitochondria preparation isolated from the cross-contamination experiments retained only 0.04% of the radioactivity. It might be possible that the difference between these two figures is attributable to the labelling of the mitochondrial RNA[16].

Electron microscopy of the mitochondria established the degree of contamination with endoplasmic reticulum. This can be recognized by negative staining of the mitochondria[27]. The integrity of the mitochondrial fraction was better evaluated by staining with ammonium molybdate since staining with potassium phosphotungstate might produce swelling and disruption of the outer mitochondrial membrane. Electron microscopic examination of a crude mitochondrial preparation negatively stained with ammonium molybdate showed some endoplasmic reticulum. This was composed mainly of smooth membranes rather than rough membranes. After repeated washings of the mitochondrial preparation no endoplasmic reticulum could be detected by electron microscopy. Thus, the biochemical and morphological evidence accumulated indicated the purity of the mitochondria. This preparation was capable of converting pregnenolone to progesterone, demonstrating the presence of the 3-β-hydroxysteroid dehydrogenase in a mitochondrial fraction devoid of microsomal contamination.

The second aim of the present study was to separate the mitochondrial membranes and to establish the location of the 3-β-hydroxysteroid dehydrogenase in submitochondrial fractions. The recently developed techniques for separation of outer and inner membranes of the mitochondria and for localization of specific enzyme markers of these membranes have been used in this study to establish the purity and the functional integrity of the submitochondrial fractions. The conversion of pregnenolone to progesterone by a lysed or sonicated mitochondrial preparation was found to be identical with an intact mitochondrial preparation. This indicated that the methods used to break the mitochondrial membranes did not influence the enzyme. On the other hand, digitonin fractionation of the mitochondria, considered to be an advantageous technique for the separation of rat liver mitochondrial membranes[23,34], could not be employed in the present experiments since the amount of digitonin required to remove the outer mitochondrial membrane inhibited the 3-β-hydroxysteroid dehydrogenase. This was probably due to the complexing ability of digitonin with pregnenolone. The subfractions obtained after differential centrifugation were examined biochemically and morphologically. The cytochrome oxidase, glutamate dehydrogenase[35] and succinic dehydrogenase[36] were selected as marker enzymes for the inner membranes and were found to be present mainly in the 105 000 $\times$ g precipitate. The monoamino-oxidase, considered to be a marker enzyme for the outer mitochondrial membrane[35] was found primarily in the 155 000 $\times$ g precipitate. The results obtained in the experiments in which rats were injected with [5-^{3}H]orotic acid showed that very little activity was associated with the submitochondrial fractions. In the cross-contamination experiment only 0.02% of the total radioactivity was found in the inner membrane fractions, whereas in the outer mitochondrial membrane no radioactivity could be detected (Table II). These results indicate that neither the inner nor the outer mitochondrial membrane were contaminated with rough endoplasmic reticulum. Electron microscopic examination of the submitochondrial fractions is in good agreement with the pattern of the marker enzymes. This confirmed that the 105 000 $\times$ g precipitate contains the inner mito-

chondrial membrane whereas the 155 000 × *g* precipitate is formed mainly from outer membrane fragments.

The bulk of 3-β-hydroxysteroid dehydrogenase activity was found to be present in the inner mitochondrial membrane. From the data illustrated in Table IV, it can be calculated that 86.5% of the total activity was localized in the inner membrane whereas 13.5% was in the outer membrane fraction. The fact that the outer mitochondrial membrane was found to contain 1/7 of the enzymatic activity, may be attributed to contamination of this fraction with inner membrane fragments.

The similar distribution of the marker enzymes did not exclude the possibility of contamination of the outer membrane with inner membrane fraction. The data accumulated in the present investigation did not permit evaluation of the degree of contamination of one membrane subfraction with the other. No attempts were made to further purify the submitochondrial fractions.

It can be concluded that the enzymes involved in the conversion of pregnenolone to progesterone are present in rat testis mitochondria, devoid of microsomal contamination. It is suggested that the inner membrane subfraction is the locus of the mitochondrial conversion of pregnenolone to progesterone. A similar localization was reported for other mitochondrial enzymes involved in steroid hormone formation, *i.e.* cholesterol side-chain cleavage enzymes[14,37] and 11β-hydroxylase[37–39].

It is assumed that the mitochondrial conversion of cholesterol to pregnenolone, catalyzed by the cholesterol side-chain cleavage enzymes, includes the rate limiting step in steroid biosynthesis which is specifically stimulated by trophic hormones (see review[40]). Pregnenolone can control its own synthesis by inhibiting the cholesterol side-chain cleavage enzymes[41,42]. It was further postulated that ACTH *via* adenosine 3′,5′-monophosphoric acid, influenced the permeability of the mitochondrial membranes and increased the rate of efflux of pregnenolone from the mitochondria to the endoplasmic reticulum. This in turn, will release the feedback inhibition and result in an overall increase in the rate of steroidogenesis[43]. The results of the present investigation indicate that in rat testis the inner mitochondrial membrane contains the enzymatic system capable of oxidizing pregnenolone to progesterone. Since progesterone is also capable of inhibiting cholesterol side-chain cleavage enzymes[44], the conversion of pregnenolone to progesterone in the mitochondria may play a part in regulating steroidogenesis.

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