DISTRIBUTION OF THE STEROID 11-β-HYDROXYLASE AND THE CYTOCHROME *P*-450 IN MEMBRANES OF BEEF ADRENAL CORTEX MITOCHONDRIA

M.SATRE and P.V.VIGNAIS
Biochimie, C.E.N.-G., Cédex 85, et Faculté de Médecine, 38 Grenoble, France

and

S.IDELMAN
Physiologie, Faculté des Sciences, 38 Grenoble, France

Received 22 August 1969

1. Introduction

Adrenal cortex mitochondria, in contrast with mitochondria from other mammalian tissues, display some morphological and biochemical analogies with endoplasmic reticulum. The adrenal cortex mitochondria are characterized by tubular or vesicular cristae [1–3] resembling somewhat the smooth endoplasmic reticulum as seen in particular in liver cells after phenobarbital injection [4]; furthermore they contain a cytochrome *P*-450 involved in the mitochondrial steroid 11-β-hydroxylation and similar to the cytochrome *P*-450 found in endoplasmic reticulum [5]. In order to assess the biochemical analogies between endoplasmic reticulum membrane and the outer mitochondrial membrane already pointed out by Parsons et al. in guinea pig liver [6] we have studied the distribution of the 11-β-hydroxylase system in membranes of beef adrenal cortex mitochondria. Enzymes localized in mitochondrial membranes and used as markers were monoamine oxidase for the outer membrane [7], malate dehydrogenase for the matrix [8], and cytochrome oxidase for the inner membrane. This report briefly describes a purification procedure of the outer and inner membranes of mitochondria from beef adrenal cortex and shows that the deoxycorticosterone 11-β-hydroxylation system and the cytochrome *P*-450 are essentially localized in the inner mitochondrial membrane.

When this manuscript was completed a paper by Yago and Ichii [9] also reported a localization of the 11-β-hydroxylase in the inner membrane of hog adrenal cortex mitochondria. These authors used a ficoll gradient to isolate mitochondrial membranes. Our results are in complete agreement with theirs and extend them by giving morphological evidence of the efficiency of the separation.

2. Methods

Beef adrenal glands were collected at a local slaughterhouse immediately after killing the animal; they were placed in crushed ice and transported to the cold room where all subsequent operations were carried out at 0–4°. The cortex was scraped from the capsule avoiding any pieces of the very hard capsule, suspended in a cold solution containing 0.27 M sucrose, 10 mM Tris-HCl and 0.2% bovine serum albumin, pH 7.4 and homogenized in a Potter-Elvejhem type glass homogenizer with a motor driven Teflon pestle at 2,000 rpm to give a 10% homogenate; we used three strokes of a loose fitting pestle and three strokes of a normal clearance pestle. The homogenate was quickly centrifuged in a Sorvall centrifuge at 1,000 × *g* for 10 min. The resulting supernatant fluid was then centrifuged at 8,000 × *g* for 15 min to sediment the mitochondria. The mitochondria were washed thrice in the sucrose-

Tris BSA medium and were finally suspended in 0.27 M sucrose buffered by 2 mM Tris-HCl, pH 7.4. 30–40 mg of mitochondrial protein were routinely obtained from one beef adrenal gland, giving a yield of about 0.8% of the wet weight of adrenal cortex tissue.

After swelling in 20 mM Na phosphate buffer, pH 7.4, subfractionation of mitochondria was carried out as described by Parsons et al. [6] with the following minor modifications. The purification gradients used with adrenal mitochondria consisted of 3 ml of 40% (w/v) sucrose, 3 ml of 30% (w/v) sucrose, 3 ml of 15% (w/v) sucrose and 1 ml of membrane suspension (from 5 to 10 mg of protein). The sucrose solutions were made in 20 mM phosphate buffer, pH 7.4. The gradients were spun in the Beckman-Spinco SW-40.1 rotor at 39,000 rpm for 1 hr. The pellet and the material gathering at the 15–30% sucrose interface were collected, diluted 4 times with phosphate buffer and sedimented by centrifugation in the Beckman-Spinco SW-40.1 rotor at 39,000 rpm for 1 hr. As shown later, these two fractions (*light and heavy*) correspond to the inner membrane + matrix and the outer membrane fractions respectively.

The beef adrenal cortex microsomes were obtained from the first mitochondrial supernatant. Mitochondria still remaining or large fragments of mitochondria were first eliminated by centrifugation at 12,000 × *g* for 20 min then the supernatant fluid was centrifuged at 105,000 × *g* for 30 min in a Beckman-Spinco centrifuge, rotor 30. The microsomal pellet was suspended in 0.27 M sucrose, 2 mM Tris-HCl, pH 7.4. When microsomes were treated with 20 mM Na phosphate in the same way as mitochondria and spun on the same sucrose gradient they gave four fractions: two minor ones, one sedimenting at the level of the first interface (input, 15% sucrose) and another at the bottom of the tube respectively, and two major ones gathering at the second and third interfaces respectively. We generally collected and re-spun the membrane fraction gathering at the second interface (between 15 and 30% sucrose) where the outer mitochondrial membrane is also found.

The cytochrome oxidase was assayed spectrophotometrically by following the oxidation of reduced cytochrome *c* [10] at pH 7.2 and 25°. Monoamine oxidase activity (MAO) was measured with [^{14}C] tryptamine as substrate according to the procedure of Wurtman and Axelrod [11] as modified by Otsuka and Kobayashi [12]. This assay offers the advantage of great sensitivity and can be used whether or not aldehyde dehydrogenase is present. Malate dehydrogenase was determined by the method of Ochoa [13]. Cytochrome *P*-450 was determined by the method of Omura and Sato [14]. To assay the 11-β-hydroxylase system, adrenal mitochondria (1–3 mg protein) were incubated 15 min at 25° in the following medium: 100 mM Tris-HCl, pH 7.4, 2 mM NADP, 20 mM glucose-6-phosphate, 2.5 μl glucose-6-phosphate dehydrogenase, 0.4 mM [^{14}C] deoxycorticosterone (0.01 μCi). The final volume was 1 ml. The steroids were extracted by 5 ml methylene chloride. [^{14}C] corticosterone was separated by thin layer chromatography [15] and counted in a Nuclear Chicago scintillation counter. More than 90% of the labeled product formed during incubation under our test conditions was accounted for by corticosterone.

The lipids were extracted by a mixture of chloroform/methanol (2:1, v/v) according to Folch et al. [16] and the lipid phosphorus determined after wet ashing according to Bartlett [17]. The protein content was measured by the biuret method [18].

Mitochondrial fractions were fixed with 1.5% glutaraldehyde in 0.1 M Na phosphate, pH 7.2 and then with 2% osmium tetroxide in the same buffer. After dehydratation by ethanol and propylene oxide specimens were embedded in Epon 812. Thin sections were stained with uranyl acetate and lead hydroxyde then examined in the Siemens Elmiskop 1A microscope. Outer mitochondrial membranes were negatively stained according to Parsons et al. [6].

3. Results

Mitochondria obtained from beef adrenal cortex showed under electron microscopic examination only slight contamination by extramitochondrial particules. They appear to be intact with an outer membrane well preserved. They display a "condensed conformation" [19] with a large vacuolar structure (fig. 1). It is noteworthy that a transition from the condensed to the "orthodox conformation" (tubulo-vesicular structure) could be obtained by incubation of the condensed mitochondria with phosphate and a respiratory substrate (succinate) as observed in rat liver mitochondria by Hackenbrock [19] (fig. 2).

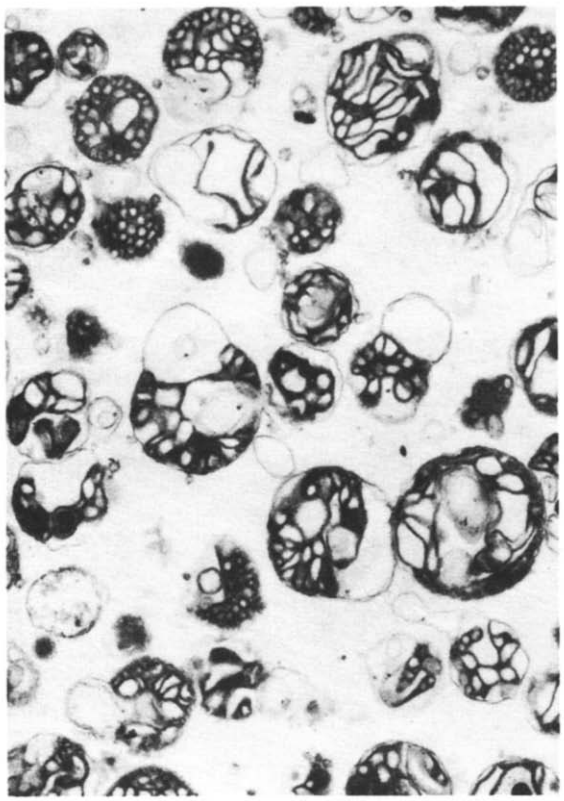

Fig. 1. Thin section of isolated mitochondria in sucrose 0.27 M, Tris 2 mM, pH 7.4. × 18,000.

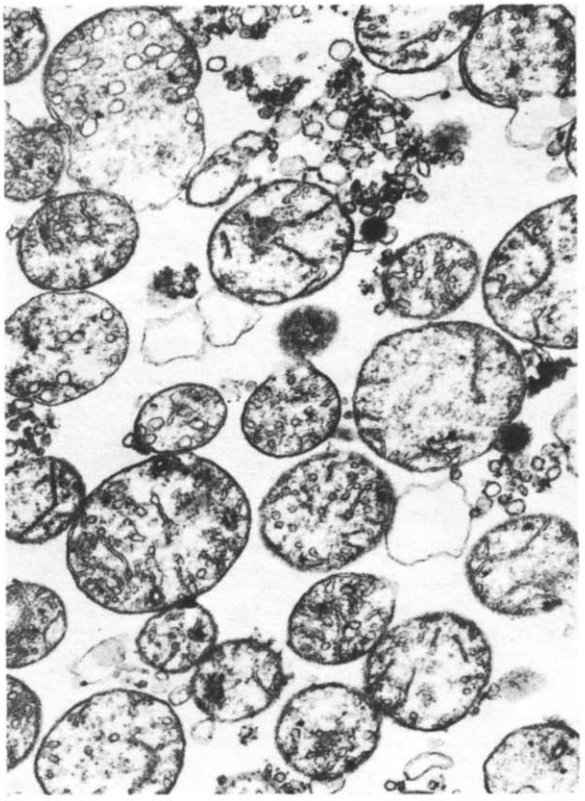

Fig. 2. Thin section of mitochondria incubated in sucrose 0.27 M, succinate 5 mM, phosphate 5 mM, pH 7.4. × 18,000.

Table 1
Characteristics of adrenal cortex subfractions.

Fraction	Density	Phospholipids**	Cyt. *c**** oxidase	Malate*** dehydrogenase	Tryptamine*** oxidase (MAO)	11-β-*** Hydroxylase	Cyt. **** *P*-450
Mitochondria	1.14	426	2,200	7,380	0.096	4.0	1.02
Inner Memb. + Matrix	1.16	461	3,270	10,400	0.102	4.4	1.31
Outer Memb.	1.08	1,130	117	140	2.57	0.55	0.09
Microsomes	–	645	316	–	0.080	0	0.39
P_i-Microsomes*	–	–	224	–	0.114	–	0.42

* Microsomes treated with orthophosphate (15–30% sucrose band) (cf. Methods).

** Phospholipids in μmoles phospholipid/mg protein.

*** Cytochrome *c* oxidase in nmoles reduced cytochrome *c* oxidized/min/mg protein; malate dehydrogenase in nmoles $NADH_2$ oxidized/min/mg protein; tryptamine oxidase in nmoles tryptamine oxidized/min/mg protein; 11-β-hydroxylase in nmoles corticosterone produced/min/mg protein.

**** Cytochrome *P*-450 in nmoles/mg protein.

As shown in table 1, the two membrane fractions obtained from adrenal cortex mitochondria exhibit significant differences bearing on phospholipid content, enzymatic activities and ability to carry out the 11-β-hydroxylation.

The lighter mitochondrial membrane fraction had a high content of phospholipid, a low cytochrome oxidase and malate NAD-dehydrogenase activities and, in contrast, a high tryptamine oxidase activity. The reverse distribution of these enzymic activities was found in the heavier membrane fraction. Since the cytochrome oxidase and the monoamine oxidase have been shown to be marker enzymes of the inner and the outer membrane respectively in rat [7] or guinea pig liver mitochondria [6], it is inferred that the lighter fraction obtained from adrenal cortex mitochondria corresponds to the outer mitochondrial membrane while the heavier fraction corresponds to the inner mitochondrial membrane.

These data were corroborated by examination under the electron microscope of either negatively stained preparations or of thin sections of these membrane fractions. Outer membranes from adrenal cortex mitochondria, negatively stained with phosphotungstate, show the same "folded bag" appearance (fig. 3) as that described by Parsons [6] in preparations of outer membrane of guinea pig liver mitochondria. The preparations of outer mitochondrial membrane are slightly contamined by smooth endoplasmic reticulum membranes recognizable by their typical tubular morphology. Actually, it was observed that when the microsomal fraction from beef adrenal cortex was treated with 20 mM Na phosphate, pH 7.4 and centrifuged in the above mentioned sucrose gradient used

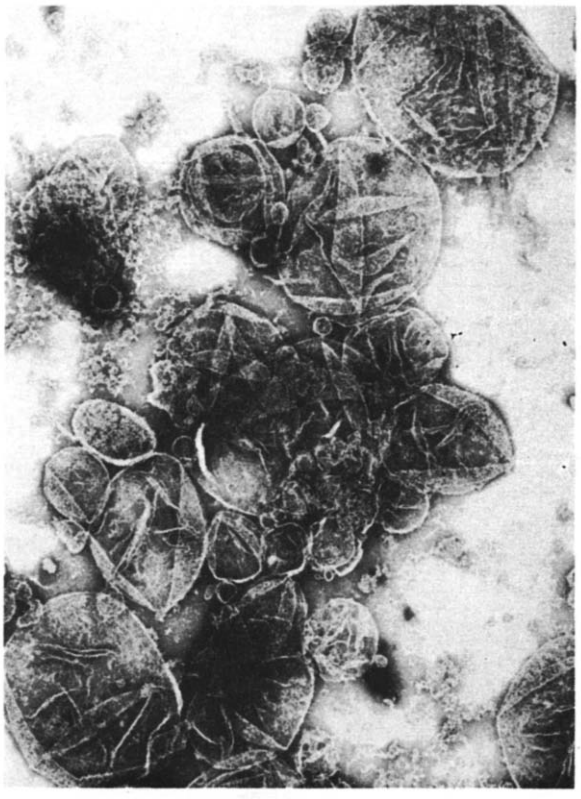

Fig. 3. Negatively stained fraction of purified outer mitochondrial membrane. X 35,000.

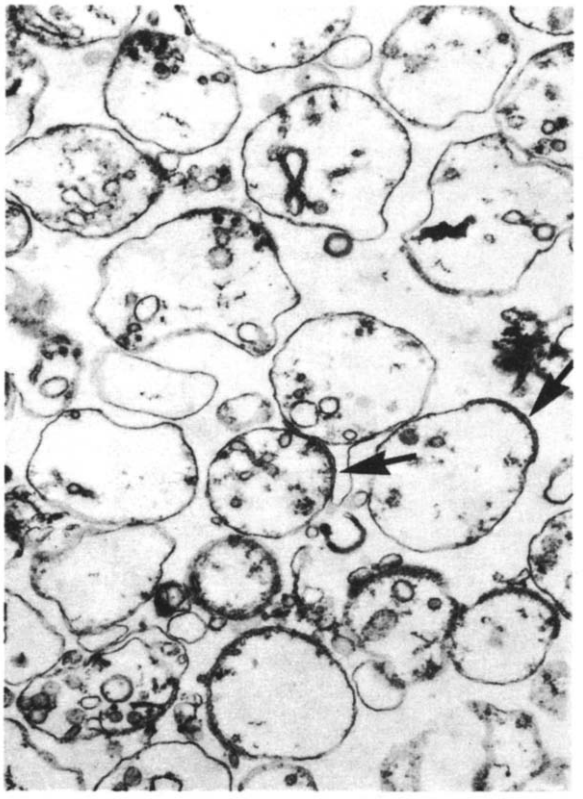

Fig. 4. Thin section of inner mitochondrial membrane fraction. The arrows point to fragments of outer membrane still attached to the inner membrane. X 18,000.

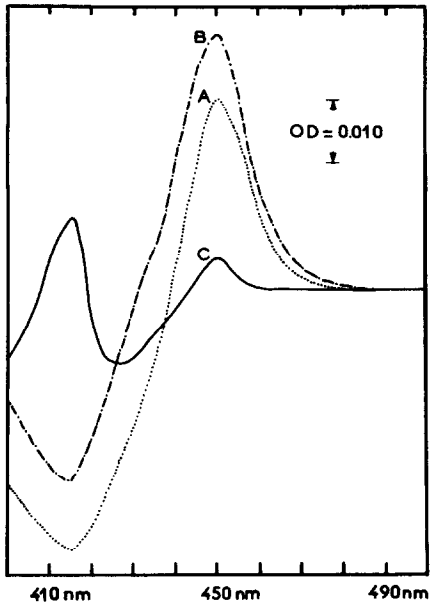

Fig. 5. Carbon monoxide difference spectra of beef adrenal cortex mitochondria (0.3 mg protein/ml; trace A), inner mitochondrial membrane (0.3 mg protein/ml; trace B), and outer mitochondrial membrane (0.6 mg protein/ml; trace C). Optical pathlength: 10 mm.

to isolate outer membrane, smooth microsomal membranes gathered at the same interface as the outer mitochondrial membranes, a result which has already been reported for guinea pig [6] and rat liver [20]. The inner membrane + matrix fraction (fig. 4) contains granulated material as it is found in similar fractions obtained from guinea pig or rat liver mitochondria [6], and some vesicles. Some fragments of outer membrane are still attached to the inner membrane but they cannot fully account for the tryptamine oxidase activity of the inner membrane fraction because the majority of inner membrane vesicles shows only one limiting membrane.

Cytochrome *P*-450 (fig. 5) and the deoxycorticosterone 11-β-hydroxylase (table 1) are selectively localized in the inner membrane fraction. The peak observed at 416 nm in the outer membrane fraction is likely to be due to adsorbed hemoglobin.

In summary, data presented in this paper show that the outer and inner membranes from adrenal cortex mitochondria can be separated in a sucrose density gradient and differentiated on the basis of *their morphology and of their enzyme distribution pattern.* It is also shown that the enzymatic system concerned with the *11-β-hydroxylation* is strictly confined to the *inner membrane (+ matrix) fraction.* The absence of steroid hydroxylase and cytochrome *P*-450 from the outer membrane is a significant difference between the outer mitochondrial membrane and the endoplasmic reticulum in beef adrenal cortex.

Acknowledgements

We wish to thank Dr. E.M.Chambaz for advice in steroid techniques and for helpful discussions.

This work was supported by the "C.N.R.S.", by the "D.G.R.S.T." and by the "Fondation pour la Recherche Médicale".

References

[1] G.E.Palade, J. Histochem. Cytochem. 1 (1953) 188.
[2] J.Luft and O.Hechter, J. Biophys. Biochem. Cytol. 3 (1957) 615.
[3] S.Idelman, Ann. Sci. Nat. Zool. 8 (1966) 205.
[4] S.J.Orrenius, J.L.E.Ericsson and L.Ernster, J. Cell. Biol. 25 (1965) 627.
[5] B.W.Harding, S.H.Wong and D.H.Nelson, Biochim. Biophys. Acta 92 (1964) 415.
[6] D.F.Parsons, G.R.Williams, W.Thompson, D.Wilson and B.Chance, in: Mitochondrial Structure and Compartmentation, eds. E.Quagliariello, S.Papa, E.C.Slater and J.M.Tager, Adriatica Editrice, Bari (1967) p. 29.
[7] C.Schnaitman, V.G.Erwin and J.W.Greenawalt, J. Cell. Biol. 32 (1967) 719.
[8] M.Klingenberg and E.Pfaff, in: Regulation of Metabolic Processes in Mitochondria, eds. J.M.Tager, S.Papa, E. Quagliariello, E.C.Slater (Elsevier Publ. Co., Amsterdam, 1966) p. 180.
[9] N.Yago and S.Ichii, J. Biochem. 65 (1969) 215.
[10] H.Beaufay, D.S.Bendall, R.Baudhuin, R.Wattiaux and C.de Duve, Biochem. J. 73 (1959) 628.
[11] R.J.Wurtman and J.Axelrod, Biochem. Pharmacol. 12 (1963) 1439.
[12] S.Otsuka and Y.Kobayashi, Biochem. Pharmacol. 13 (1964) 995.
[13] S.Ochoa, in: Methods in Enzymology, vol. 1, eds. S.P. Colowick and N.O.Kaplan (Academic Press, New York, 1955) p. 735.
[14] T.Omura and R.Sato, in: Methods in Enzymology, vol. 10, eds. S.P.Colowick and N.O.Kaplan (Academic Press, New York, 1967) p. 556.

[15] R.Neher, in: Steroid Chromatography (Elsevier, Amsterdam, 1964) p. 250.
[16] J.Folch, I.Ascoli, M.Less, J.Maeth and F.Le Baron, J. Biol. Chem. 191 (1954) 833.
[17] G.R.Bartlett, J. Biol. Chem. 234 (1959) 466.
[18] A.G.Gornall, C.S.Bardawill and M.M.David, J. Biol. Chem. 177 (1949) 751.
[19] C.R.Hackenbrock, J. Cell. Biol. 37 (1967) 345.
[20] P.M.Vignais and J.Nachbaur, Biochem. Biophys. Res. Commun. 33 (1968) 307.