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ELECTROPHORETIC ANALYSIS AND CYTOCHROME *P*450 CONTENTS OF BOVINE ADRENAL CORTEX MITOCHONDRIA AND OF THEIR SUBFRACTIONS

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SUMMARY

Adrenal cortex mitochondria were fractionated into outer membrane and inner membrane. This second membrane was further separated into water-soluble proteins, KCl extractable proteins, "structural proteins" and structured cytochromes. Cytochrome *P*450 located in the inner membrane was found only in the structured cytochrome fraction. The protein components of all the fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Mitochondria contain at least 20 components ranging in molecular weight from 10,000–120,000 daltons. The outer membrane is different from the inner membrane both in number of components and size distribution. In structured cytochrome proteins a component of 52,000 molecular weight represents about 25% of the whole protein contents. This corresponds to the subunit of cytochrome *P*450. Proteins synthesized in isolated adrenal cortex mitochondria in the presence of radioactive leucine reveal six groups of labelled polypeptides when subjected to polyacrylamide gel electrophoresis. The molecular weights of these proteins ranged from 10,000–54,000 daltons. In the fraction of structured cytochromes a labelled component having a molecular weight 52,000 daltons was found. It is thought that this component may correspond to the subunit of cytochrome *P*450 and be coded partly by mitochondrial nucleic acids.

INTRODUCTION

Mitochondria from the adrenal cortex possess, in contrast to other mitochondria from mammalian tissues, two distinct types of respiratory chains. One is similar to the respiratory chain observed in liver, heart or muscle mitochondria, the other is functional for the 11β, 18, 20 and 22 hydroxylations [1, 2]. This hydroxylating chain consists of an NADPH-dependent flavoprotein (adrenodoxin reductase), a non-heme iron protein (adrenodoxin) and a cytochrome *P*450 which is a hemoprotein [3–5]. Cytochrome *P*450 serves as the terminal oxygenase by transferring electrons from NADPH to oxygen. It has been isolated in a form having a molecular weight of 850,000 daltons dissociated in 16 subunits, each having a molecular weight of 52,000 daltons [6, 7].

Mitochondria contain DNA and are able to synthesize some proteins [8] located in the inner membrane [9]. The mitochondrial genome participates in the biosynthesis of several enzymes such as ATPase, cytochrome oxidase and cytochrome *b* [10–12]. It has also been shown that when isolated bovine adrenal cortex mitochondria were incubated with radioactive amino acid, a labelled protein with a mobility similar to that of adrenodoxin was present [13]. Cytochrome *P*450 has been characterized as a component of the inner membrane [14, 15] but it is not yet known in what fraction isolated from inner membrane this cytochrome is present. Furthermore, nothing is known about where its synthesis takes place in mitochondria or extramitochondria.

In the present work, cytochrome *P*450 was characterized as a specific subfraction of inner mitochondrial membrane. Furthermore, proteins of whole mitochondria, the two membranes and the inner membrane components were analyzed by polyacrylamide gel electrophoresis. Finally, the size distribution of the products synthesized *in vitro* in the adrenal cortex mitochondria proteins was examined to determine if the protein containing cytochrome *P*450 depends on mitochondrial nucleic acids.

MATERIAL AND METHODS

Preparation of mitochondria. Bovine adrenal glands were brought in ice to the laboratory within 1 h after slaughter of the animals. Mitochondria were prepared according to the method of Cammer and Estabrook [1]. The glands [10–15] were bisected, the medulla carefully removed and after scraping from the capsule, the cortex was homogenized in 0.25 M sucrose. The homogenate was centrifuged twice at 900 *g* for 10 min and at 9000 *g* for 10 min more. The sedimented mitochondria were washed three times in 0.25 M sucrose and again centrifuged at 9000 *g* for 10 min.

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The quality of mitochondria was controlled by measuring oxidative phosphorylation properties and the activity of the 11β-hydroxylase [16]. The respiration and the phosphorylation of mitochondria were recorded by an oxygraph GME with an oscillating platinum electrode capped with a polythene membrane. The mixing buffer (pH 7.4) had the following composition: 0.25 M sucrose, 20 mM KCl, 10 mM phosphate buffer at pH 7.4, 15 mM triethanolamine 5 mM $MgCl_2$ [1]. The substrate used was malate in the presence of malonate. The activity of the 11β-hydroxylase was determined by measuring corticosterone formed during the incubation of mitochondria with deoxycorticosterone. The corticosterone synthesized was measured fluorometrically by the method of Mattingly [17].

Fractionation of mitochondria. Mitochondria were fractionated into outer membrane and mitoplast (inner membrane and matrix) according to the procedure of Parsons [18]. Particles were treated with hypotonic solution. The outer membrane and the mitoplast were separated by differential centrifugations and purified. The mitoplast was fractionated according to the procedure described by Beattie *et al.* for liver [19]. Mitoplast was extracted with water for 5 min at 30° to remove the water-soluble proteins. The insoluble material represented the inner membrane, the purity of which was controlled by the determination of the activity of cytochrome *c* oxidase. The activity of this enzyme was estimated according to a polarographic procedure by determining the oxidation of an ascorbate solution in the presence of N,N,N′,N′-tetramethylenediamine [20].

The inner membrane was then extracted with 0.15 and 0.6 M KCl to remove cytochrome *c* and the other extrinsic proteins. The residue was treated with sodium cholate, sodium deoxycholate and sodium dodecylsulphate. The solution was brought to 13% saturation with neutralized saturated ammonium sulphate for "structural proteins" and to 50% saturation with ammonium sulphate to yield a fraction containing cytochromes. Protein analysis were carried out using the technique described by Lowry *et al.* [21] and Gornall *et al.* [22].

Spectrophotometric determination of cytochrome P450. Cytochrome *P*450 was determined according to Omura and Sato by differential spectra [23]. Mitochondria proteins (about 2 mg) were suspended in 1 ml 0.1 M phosphate buffer pH 7.0. A few crystals of sodium dithionite were added in order to reduce possible blood pigment traces. The contents of one cuvette were then gassed with CO which forms a "*P*450–CO" complex. The concentration of *P*450 can be calculated by using the extinction coefficient differences between 450 (peak) and 490 nm which is 91 $cm^{-1} \cdot mM^{-1}$.

Over diluted soluble proteins and KCl extractable proteins were concentrated by ultrafiltration through Diaflo membranes. Their cytochrome *P*450 contents were then determined.

Polyacrylamide gel electrophoresis. Electrophoresis was performed in polyacrylamide gel (8.5% total acrylamide concentration, 0.22% methylene bisacrylamide to acrylamide, 0.075% ammonium peroxodisulphate and 0.15% tetramethylenediamine) in 0.01 M sodium phosphate (pH 7.0) with 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol. Samples (50–100 μg protein) dissolved in the buffer used for electrophoresis were submitted to a constant current of 8 mA/tube for 6 h. Gels were stained overnight with 0.04% Coomassie brilliant blue (w/v), 45% methanol (v/v), 9% acetic acid (v/v) and destained with 5% methanol, 7% acetic acid [24]. Photometric tracing of the stained gels was performed in a Gilford gel spectrophotometer. Molecular weights were determined by using commercial marker proteins [25]: β-galactosidase (130,000), phosphorylase a (94,000), bovine serum albumine (68,000), creatine kinase (40,000), trypsin (23,300) and cytochrome *c* (11,700).

[^3H]-Leucine incorporation into mitochondrial proteins. Adrenal cortex mitochondria (2 mg) were incubated *in vitro* for 20 min in a metabolic shaker at 30° in 2 ml of medium containing: 50 mM Bicine buffer pH 7.6, 10 mM $MgCl_2$, 5 mM KH_2PO_4 pH 7.6, 90 mM KCl, 22.5 μg/ml of a complete amino acid mixture, minus leucine, 100 μCi of L-[4,5-^{3}H]-Leucine, S.A. 60 Ci/mmol (Amersham) with an artificial system generating ATP formed by 2 mM ATP, 5 mM phosphoenol pyruvate and 20 μg pyruvate kinase [26]. The reaction was stopped by adding cold 0.25 M sucrose containing an excess of unlabelled leucine. Mitochondria were again isolated at 9000 *g* for 10 min and washed twice with 0.25 M sucrose and then fractionated according to the method of Beattie *et al.* [19]. The different mitochondrial subfractions were precipitated with 10% trichloracetic acid and afterwards they were dissolved for electrophoretic analysis. The electrophoresis of each sample was performed in duplicate, one was stained and the other was cut into 1.0 mm slices using a Gilson model gel slicer. The slices were burned in an Oxidizer Tricarb (model 306 Packard). The radioactivity was counted in a Packard liquid scintillation counter.

RESULTS

Submitochondrial distribution of cytochrome P450

The cytochrome *P*450 contents estimated in adrenal cortex mitochondria and in their different subfractions are given in Table 1. In the intact mitochondria, the concentration of cytochrome *P*450 is 0.96 nmol/mg protein. The outer membrane and the water-soluble proteins are devoid of cytochrome *P*450; all the cytochrome is included in the inner membrane with a value of 1.20 nmol/mg protein. At the same time, the cytochrome *c* oxidase activities in the whole mitochondria and in the inner membrane are 217 and 389 nmol $O_2 \cdot mn^{-1} \cdot mg^{-1}$ protein (Table 1). The KCl extractable proteins and the "structural proteins" do not contain cytochrome *P*450

Table 1. Cytochrome *P*450 contents and cytochrome *c* oxidase activity in adrenal cortex mitochondria and in their subfractions

Fractions	Cytochrome *c* oxidase (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ prot.)	Cytochrome *P*450 (nmol $\cdot mg^{-1}$ prot.)
Whole mitochondria	217 ± 19*	0.96 ± 0.15*
Outer membrane		Not detectable
Inner membrane	389 ± 35*	1.20 ± 0.16*
Water-soluble proteins		<0.01
KCl-soluble prot. (0.12 M)		<0.01
KCl-soluble prot. (0.60 M)		<0.01
"Structural proteins"		<0.01
Cytochromes		*P*420 *P*450
		0.88 1.24
Supernatant		<0.01

Each of the values represents the average of six experiments.
* Mean values ± S.E.

which is entirely located in the structured cytochrome fraction. In this fraction, obtained after the proteins had been dispersed by detergents, the cytochrome *P*450 has an absorption both at 450 and 420 nm. As shown in Fig. 1 the spectra of cytochrome *P*450 in the inner membrane, before the action of detergents, indicates one peak at 450 nm. However, as previously reported [27], two peaks appear at 450 and 420 nm, after the action of detergents.

Electrophoresis

The method of electrophoresis used in these experiments, resolves the mitochondrial proteins into more than 20 major constituents ranging in molecular weight from 10,000–120,000 daltons, as is shown in the densitometric pattern (Fig. 2a). Cytochrome *c* used as an internal reference makes it possible to establish very specific relative migrations. In the electrophoretic conditions adopted here, the migration mainly depends on the size of particles as proved with

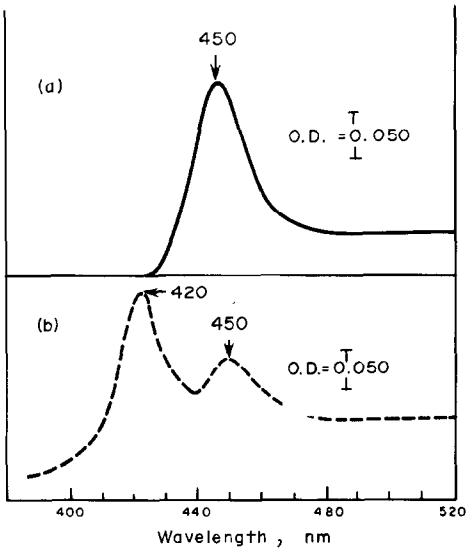

Fig. 1. Carbon monoxide difference spectra of untreated (a) and detergent-treated inner membrane (b). O.D.: optical density.

differencial commercial marker proteins. By comparison, it was possible to estimate the molecular weights of the protein components. The electrophoretic pattern of the outer membrane proteins is given in Fig. 2b. As regards this fraction, the number of bands is clearly fewer than that of whole proteins, the majority having a molecular weight lower than 64,000 daltons. The electrophoretic profile of the mitoplast (Fig. 2c) is very similar to that of whole mitochondria, which can be explained by the fact that this fraction includes more than 85% of all mitochondrial proteins. The inner membrane electrophoretic pattern (Fig. 2d) is also similar to that of whole mitochondria. However, it must be considered that the band of molecular weight about 60,000 daltons is decreased by 20% compared with intact mitochondria. Moreover, this band is 2.5-fold increased in the water-soluble proteins and in the KCl extractable proteins (Fig. 3a and 3b). In these two fractions a decrease of about 50% can also be seen in the components with molecular weights lower than 40,000 daltons. In contrast these same proteins are increased 2-fold in the "structural proteins" (Fig. 3c) and in addition, the protein components with molecular weights of 38,000 and 28,000 daltons are increased in this fraction. Lastly, in the structured cytochrome fraction (Fig. 3d) the protein of a molecular weight of 56,000 daltons is markedly decreased while the band with a molecular weight of 52,000 daltons is a major component, representing about 27% of the total protein. Evidence suggests that it corresponds to the cytochrome *P*450 subunit.

Protein biosynthesis

The proteins synthesized in adrenal cortex mitochondria in the presence of tritiated leucine as labelled amino acid, are principally located in the inner membrane as is noted after separation of mitochondria into water-soluble proteins and inner membrane. The distribution of radioactivity in the inner membrane after electrophoresis reveals labelled components ranging in molecular weights for the most part from 20,000 to 34,000 daltons. Three less important peaks corresponding to molecular weights

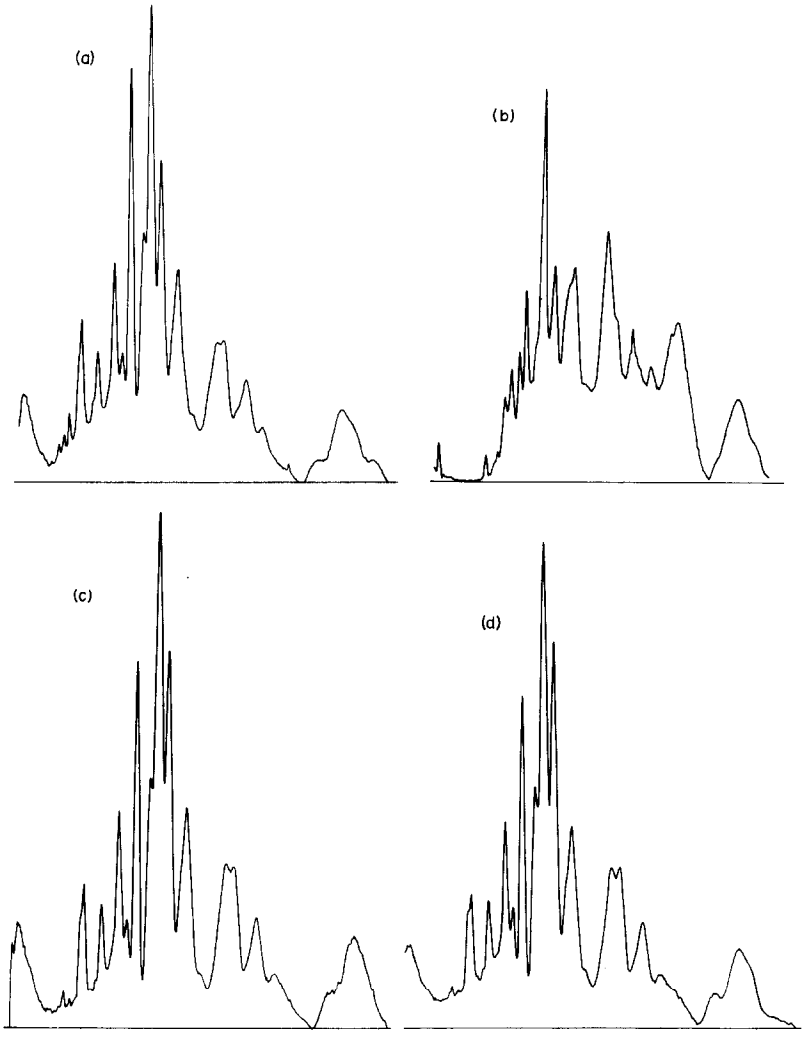

Fig. 2. Densitometric traces of polyacrylamide gel electrophoresis of adrenal cortex mitochondria proteins. (a): whole mitochondria; (b): outer membrane; (c): mitoplast; (d): inner membrane.

54,000, 42,000 and 10,000 daltons are also present (Fig. 4). The fractionation of the inner membrane shows that the "structural proteins" are labelled twice as much as those of the cytochrome fraction. The distribution of radioactivity in the "structural proteins" is similar to that of the inner membrane (Fig. 5). The radioactivity electrophoretic pattern of the cytochrome fraction is shown in Fig. 6; in this case, the distribution of radioactivity is of a wider range. However, the distribution is also present in the same areas as previously shown. It must be noted that some of the radioactive amino acid is incorporated into proteins of a molecular weight of 52,000 daltons in which cytochrome *P*450 is present.

DISCUSSION AND CONCLUSION

Data reported in Table 1 show that whole adrenal cortex mitochondria contain about 0.96 nmol of cytochrome *P*450 per mg protein. After removal of outer membrane and soluble proteins, the cytochrome *P*450 concentration reaches 1.20 nmol/mg protein. This increase of about 25% can be explained by the lack of this cytochrome in the discarded soluble proteins. The activity of cytochrome *c* oxidase is also higher in the inner membrane mitochondria than in whole mitochondria. The fractionation of the inner membrane according to the procedure previously described for liver mitochondria [19] can be applied to adrenal cortex mitochondria to obtain KCl extractable proteins, "structural proteins" and a fraction containing structured cytochromes. The determination of cytochrome *P*450 in these different fractions indicates the lack of this cytochrome in KCl extractable proteins and "structural proteins"; it was found only in the fraction of structured cytochromes. It has been confirmed that this fraction contains cytochromes *b*, c_1, *a* and a_3, and it is similar to that identified in liver mitochondria. As the structured cytochrome fraction of adrenal cortex mitochondria includes both cytochrome oxidase which is the terminal component of the respiratory chain, and cytochrome *P*450 which is the terminal oxygenase of the hydroxylating chain, it is suggested that the use of oxygen is similar in both these processes.

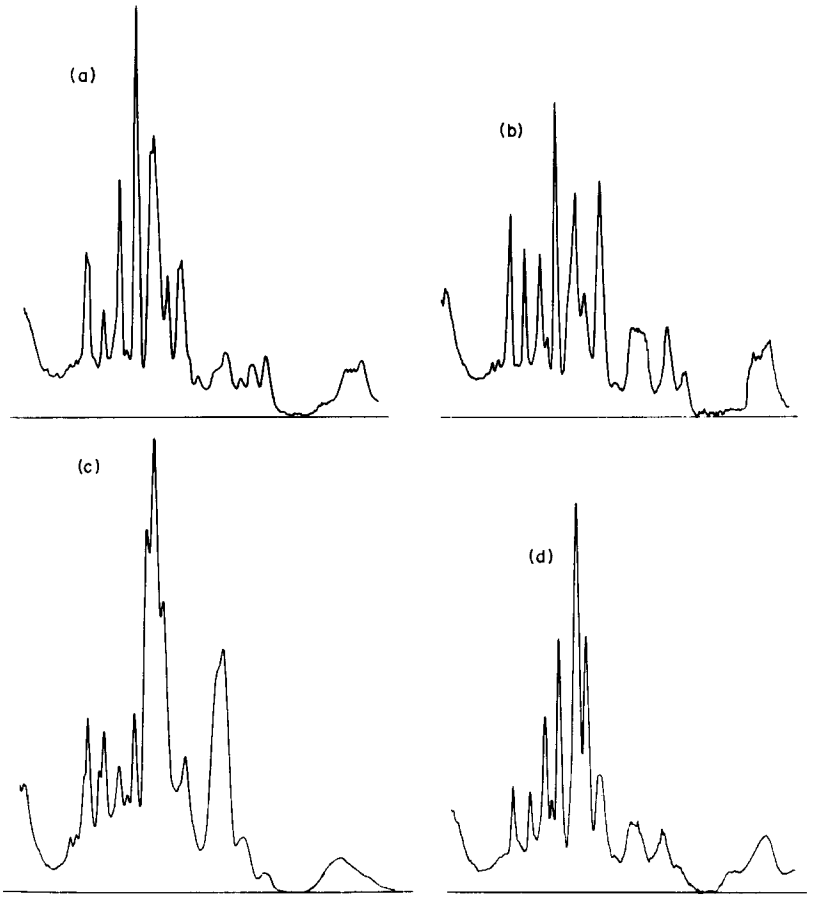

Fig. 3. Densitometric traces of polyacrylamide gel electrophoresis of proteins of inner membrane subfractions. (a): water-soluble proteins; (b): KCL-soluble proteins; (c): "structural proteins"; (d): Cytochrome proteins.

Electrophoresis of intact mitochondria shows a complex pattern involving about 20 components which are also found but in variable amounts in the different particular subfractions. The electrophoretic profile of the outer membrane consists of about 12 major components. It has considerable similarity to the densitometric pattern obtained from the corresponding liver fraction [28]. Electrophoresis of matrix

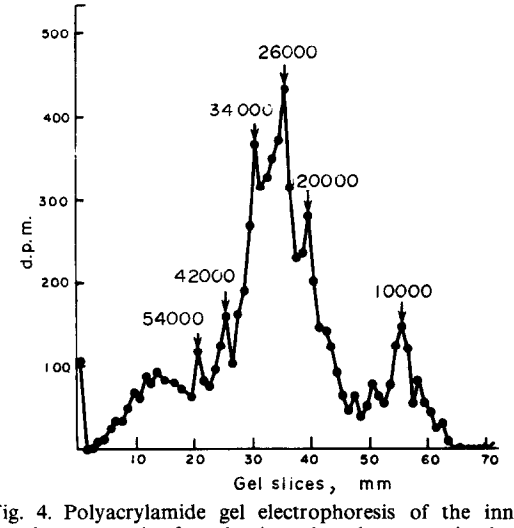

Fig. 4. Polyacrylamide gel electrophoresis of the inner membrane proteins from bovine adrenal cortex mitochondria, labelled *in vitro*. The mitochondria were disrupted in water and the inner membrane centrifuged down at 9000 *g* for 10 min. The molecular weights indicated for peaks in the figure were calculated according to marker proteins.

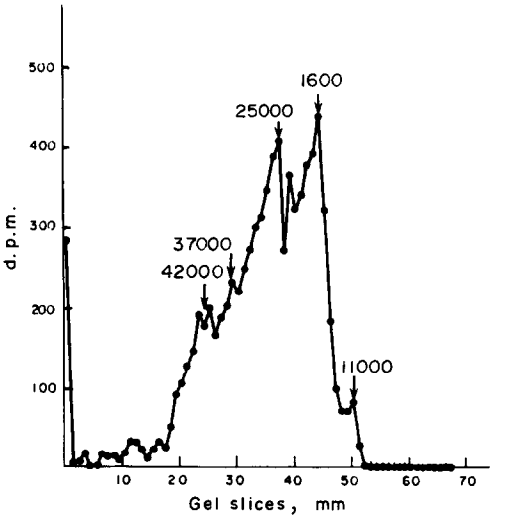

Fig. 5. Polyacrylamide gel electrophoresis of the "structural proteins" of inner membrane mitochondria, labelled *in vitro*. The molecular weights for peaks are indicated in the figure.

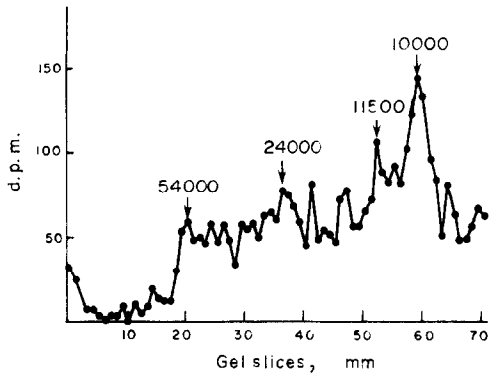

Fig. 6. Electrophoretic profile of radioactivity of the structured cytochrome proteins. The molecular weights for peaks are indicated in the figure.

soluble proteins on the one hand and "structural proteins" and KCl extractable proteins on the other also shows similar profiles to those obtained with corresponding liver fractions [29]. The structured cytochrome fraction is made up of 6 major components, one of which represents more than 25% of the whole protein content and has a molecular weight of 52,000 daltons. It must be noted that cytochrome *P*450 consists of 16 subunits, each having a molecular weight of 52,000 daltons [7]. The result strongly suggests that cytochrome *P*450 is a part of this component, but that it evidently represents only a small percentage of it. It is possible that the peak found for this component represents several polypeptides, since, in the fraction where cytochrome *P*450 is lacking, for example in the water-soluble proteins, the peak is present but it is less pronounced.

The incorporation of labelled amino acid in isolated liver mitochondria has been the subject of much investigation [30, 31], but not so for adrenal cortex mitochondria. Up to now, only Asano *et al.* have shown that these particles exhibit incorporation properties similar to those of liver mitochondria. These workers have characterized a soluble labelled protein having a molecular weight of 50,000 daltons which is thought to be adrenodoxin [13]. Our present work shows that labelled leucine is incorporated into several proteins. The electrophoretic separation shows at least 6 groups of labelled proteins having molecular weights ranging from approximately 10,000–54,000 daltons. The fractionation of labelled mitochondria shows that all the radioactivity is located in the inner membrane as is the case in liver particles [9]. Subfractionation and electrophoresis of this membrane show a specific radioactivity which is higher in the "structural proteins" than in the structured cytochrome fraction. In the inner membrane, the peak with a molecular weight of 52,000 daltons is labelled whereas in the outer membrane this peak is unlabelled as previously mentioned. It can be supposed that the same proteins which are found in the outer membrane are also found in the inner membrane where they are unlabelled. It can therefore be suggested that the labelled part corresponds to cytochrome *P*450. It is therefore possible that the cytochrome *P*450 which is the oxygenase of the hydroxylating chain depends partly on mitochondrial nucleic acids as was found to be the case for cytochrome oxidase which is the oxygenase of the respiratory chain [10].

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