Biochemical Aspects of the Biogenesis of Mitochondria

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CONTENTS

INCORPORATION OF AMINO ACIDS INTO THE OUTER AND INNER MEMBRANE OF ISOLATED RAT-LIVER MITOCHONDRIA

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I would like to give first a brief review of the recent developments in the field of protein synthesis in isolated mitochondria. My discussion shall be confined to those papers which have appeared since the 1965 meeting in Bari « On the Regulation of Metabolic Processes in Mitochondria » (refs. 1-4; see also ref. 5).

An interesting article is that of Wheeldon and Lehninger [6]. In the hands of these authors the incorporation of radioactive amino acids is supported by ATP and an ATP-generating system. Incorporation driven by this system appears to be far more rapid than when it is supported by electron transport. The authors discuss the complexity of the role of ATP which appears to be involved in many steps of the incorporation process. Furthermore they found that oligomycin does not inhibit the ATP supported incorporation, indicating that « high-energy intermediates of oxidative phosphorylation generated by electron transport or ATP hydrolysis are not obligatory in providing energy for mitochondrial protein biosynthesis ». This is in some contrast to previous findings where sensitivity to oligomycin led to the suggestion that high-energy intermediates may be involved in the incorporation process [7, 8]. One other aspect of this paper is the study of the decay of the newly synthesized proteins. Wheeldon and Lehninger [6] have found that acid-soluble neutral peptides are formed by this enzymatic decay, and that this

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process is stimulated by puromycin and GTP, whereas it is inhibited by antimycin A.

Many of the findings obtained with rat-liver and beef-heart mitochondria have also been obtained with mitochondria from Krebs ascites tumor cells [9], plant tissue [10], beef adrenal cortex [11], cerebral cortex and spinal cord of the rat [12].

There are only few papers which are concerned in more detail with the product of protein synthesis in isolated mitochondria. Haldar *et al.* [13] have shown that the so-called « structural protein » prepared from rat-liver mitochondria and which had been labelled *in vitro* with radioactive valine, showed several radioactive bands when separated by polyacrylamide gel electrophoresis. They suggested that one of these radioactive bands corresponds to Racker's coupling factor 4. O'Brien and Kalf [14] have recently reported that ribonucleic particles isolated from rat-liver mitochondria after incubation with [^{14}C] leucine *in vitro*, contain a specific radioactivity which is 50 times higher than that of the total mitochondrial protein.

Thus although the conditions influencing the incorporation of amino acids into isolated mitochondria are now quite well understood, little is known regarding the nature and location of the product of mitochondrial protein biosynthesis. Is has been established that the radioactive amino acids incorporated by isolated mitochondria are associated almost entirely with the insoluble membrane proteins [1, 6, 15-17]. In an attempt to further localize the product and the site of the incorporation of amino acids, we have made use of the recently reported techniques of separating the inner and outer membranes of rat-liver mitochondria [18-22, 25].

Incorporation of radioactive leucine into isolated intact rat-liver mitochondria followed by separation of outer and inner membrane

Mitochondria were incubated for 40 min at 32° in a medium containing: 0.25 M sucrose, 20 mM KCl, 15 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM ADP, 2 mM EDTA, 0.188 mg/ml amino acid mixture, 0.06 mg/ml streptomycin, 0.02% bovine serum albumin, 4-5 mg/ml mitochondrial protein and 0.3 μC/ml [^{14}C] l-leucine (325 mC/mmole). The pH was 7.6, and the final volume 7 ml. Following incubation, the mitochondria were washed by cen-

trifugation. The inner and outer membranes were then prepared by one of the following procedures:

(a) a slightly modified « swelling-shrinking » procedure combining the methods of Parsons *et al.* [18, 19] and of Sottocasa *et al.* [20];

(b) the « digitonin » method of Schnaitman *et al.* [22].

In the first method the labelled mitochondria were made to swell by exposure to a hypotonic medium (20 mM phosphate buffer). After 10 min 2 mM ATP and concentrated sucrose were added to shrink the inner membranes and to make the suspension isotonic. A high-speed centrifugation (35 000 x *g*) sheared off the outer membranes from the inner membranes. A low-speed centrifugation (1900 x *g*) then removed a large part of the inner-membrane ghosts. The supernatant mitochondrial fragments were separated on a discontinuous density gradient as described by Parsons *et al.* [19]. Two bands (fractions LI and LII) and a pellet (fraction LIII) were obtained. LI is that fraction in which the outer membrane is enriched, LII and LIII are those fractions in which the inner membrane is enriched. The fractions were followed with enzymic markers. Monoamine oxidase was routinely used as an outer membrane marker [22], and succinate-cytochrome *c* reductase as a marker for the inner membrane [20]. Table I shows the distribution of succinate-cytochrome *c* reductase and monoamine oxidase in the different fractions of five « swelling-shrinking » preparations. The purities reported by Parsons *et al.* [19] were not obtained in these preparations, since, as already found by these workers, the separation is badly influenced by conditions such as our 40-min exposure at 32°. Also we have not routinely recentrifuged the single bands. The distribution of the specific radioactivities is also shown in Table I.

It is obvious that in fraction LI, outer membrane is contaminated by inner membrane, and that in fractions LII and LIII inner membrane is contaminated by outer membrane. Therefore, the proportions of « pure » outer and « pure » inner membrane in each fraction had to be calculated. This was done on the basis of marker enzyme activities. The principle of this calculation is as follows:

$$p_{oLI} + p_{iLI} = 1$$

$$p_{oLII} + p_{iLII} = 1$$

TABLE I

Specific activities of succinate-cytochrome c reductase and monoamine oxidase and specific radioactivities in different fractions of mitochondria prepared by the « swelling-shrinking » method, following incorporation of [^{14}C]-l-leucine

Abbreviations: SDH, succinate-cytochrome c reductase; MAO, monoamine oxidase; SR, specific radioactivity of protein; MS, mitochondrial suspension; LM, input to density gradient; LI, upper fraction of gradient; LII, middle fraction of gradient; LIII, pellet. Enzyme activities are expressed in μmoles/h per mg protein, specific radioactivities in counts/min per mg protein.

Fraction	Activity	μmoles/h or counts/min (per mg protein) in Expt.				
		1	2	3	4	5
MS	SDH	—	9.8	14.5	—	7.7
	MAO	—	803	224	—	595
	SR	—	112	44	—	117
LM	SDH	21.2	38.0	28.4	27.6	25.2
	MAO	1800	2240	1480	785	1570
	SR	81	238	126	248	180
LI	SDH	7.8	7.0	11.6	2 1	12.5
	MAO	3340	2960	3180	4310	3855
	SR	26	83	66	50	122
LII	SDH	29.6	18.3	40.4	24.8	23.0
	MAO	1320	1035	962	803	1210
	SR	102	240	238	361	244
LIII	SDH	40.2	25.4	24.7	18.8	27.1
	MAO	698	562	328	206	1050
	SR	104	168	85	88	192
SR of pure outer membrane (A)		0.3	14	2	21	—18
SR of pure inner membrane (B)		151	325	314	439	294
A as % of B		0.2	4.2	0.6	4.8	—6.0

where p_{oLI} and p_{oLII} represent the proportions of « pure » outer membrane in fractions LI and LII, respectively, and where p_{iLI} and p_{iLII} represent the proportions of « pure » inner membrane in fractions LI and LII, respectively.

Now $$p_{oLI} = \frac{E_{MAO\ LI}}{E_{MAO\ po}}\ ;\quad p_{iLI} = \frac{E_{SDH\ LI}}{E_{SDH\ pi}}\ ;$$

$$p_{oLII} = \frac{E_{MAO\ LII}}{E_{MAO\ po}}\qquad p_{iLII} = \frac{E_{SDH\ LII}}{E_{SDH\ pi}}\ ;$$

where $E_{MAO\ LI}$, $E_{MAO\ LII}$, $E_{SDH\ LI}$, $E_{SDH\ LII}$ represent the specific activities of monoamine oxidase and succinate-cytochrome *c* reductase in bands LI and LII respectively. $E_{MAO\ po}$ represents the specific activity of MAO in pure outer membrane and $E_{SDH\ pi}$ represents the specific activity of SDH in pure inner membrane.

The specific radioactivities of pure outer and inner membrane can be calculated by the following equations:

$$p_{oLI} \cdot SR_{po} + p_{iLI} \cdot SR_{pi} = SR_{LI}.$$

$$p_{oLII} \cdot SR_{po} + p_{iLII} \cdot SR_{pi} = SR_{LII}.$$

where SR_{po}, SR_{pi}, SR_{LI}, SR_{LII} represent the specific radioactivities of pure outer membrane, pure inner membrane, fraction LI and fraction LII, respectively.

It is necessary for the application of this calculation that the membrane proteins in each fraction are not contaminated with non-membraneous proteins. As shown in Table II this is only true, at least to a certain degree, for fractions LI and LII. The specific activities of malate dehydrogenase and glutamate dehydrogenase indicate a reasonably high proportion of so-called « soluble » matrix proteins in fraction LIII. In fractions LI and LII the proportions of these enzymes are low and similar. Therefore, only these fractions were used for the calculation of the specific radioactivities of « pure » outer and inner membrane according to the described method.

The results of this calculation are shown in Table I. The

TABLE II

Specific activities of some « insoluble » and « soluble » enzymes in different fractions of mitochondria prepared by the « swelling-shrinking » method

Abbreviations: MDH, malate dehydrogenase; GluDH, glutamate dehydrogenase. Enzyme activities are expressed in μmoles/h per mg protein.

Fraction	Specific activities (μmoles/h per mg protein)			
	SDH	MAO	MDH	GluDH
MS	8.0	549	220	201
LM	11.2	1260	41	61
LI	3.4	4460	37	46
LII	12.0	597	69	69
LIII	20.8	302	265	286

specific activity of the « pure » outer membrane appears to be less than 5% of that of the « pure » inner membrane.

Let us now discuss the digitonin procedure. The basic principle of this method is that a relatively low concentration of digitonin (1.0 — 1.1 mg/10 mg mitochondrial protein) preferentially solubilizes the outer membrane [21, 22]. Small vesicles are formed which can be separated by high-speed centrifugation. The experiments of Schnaitman *et al.* [22] show that the digitonin concentration for obtaining optimal separation of outer and inner membranes is very critical. They also show that the low-speed pellet (9500 x *g*), which contains the bulk of the inner-membrane ghosts, has a high proportion of soluble non-membrane-bound enzymes. Therefore, this fraction is not suitable for our method of calculation. The succinate-cytochrome *c* reductase and monoamine oxidase activities in the 35 000 x *g* and 144 000 x *g* pellets which were used do not differ very much. This means that the limit of error in this type of fractionation is much higher. However, again the specific radioactivity of the « pure » outer membrane is low when compared with that of the « pure » inner membrane (Table III).

The method of calculation which is used here appears to be pertinent provided the following assumptions are valid: First, the enzyme activities of monoamine oxidase and succinate-cytochrome *c* reductase are specific for outer and inner membrane respectively. The experiments of Parsons *et al.*, Schnaitman *et al.* and Sottocasa *et al.* would indicate this assumption is true. However, we would like to stress the possibility that within the various fractions, different proportions of different parts of the inner membrane may be present, *i.e.* the building sites into which the radioactive amino acids are incorporated may be distributed in the gradient in a pattern different to that of the succinate-cytochrome *c* reductase-carrying cristae fragments. We shall discuss later some evidence for this possibility. Secondly, the turnover times of the outer and inner membrane must be the same. There is little information available with respect to this point, but the following experiments shed some light on it. Fig. 1 shows the distribution of the insoluble proteins of locust flight-muscle mitochondria after separation by electrophoresis on polyacrylamide gel. Locusts were injected with radioactive isoleucine. After 5 h the animals were killed and the insoluble mitochondrial proteins prepared. Fig. 2 represents

TABLE III

Specific activities of succinate-cytochrome c reductase and monoamine oxidase, and specific radioactivities in different fractions of mitochondria prepared by the digitonin method, following incorporation of [^{14}C] l-leucine

Abbreviations and activities are expressed as in Table I.

Fraction	Activity	μmoles/h or counts/min (per mg protein)	
		Expt. 1	Expt. 2
MS	SDH	—	7.2
	MAO	—	644
	SR	79	65
9 500 x *g* pellet	SDH	31.6	26.2
	MAO	548	711
	SR	194	70
35 000 x *g* pellet	SDH	5.5	18.7
	MAO	568	1190
	SR	97	96
144 000 x *g* pellet	SDH	2.0	12.6
	MAO	855	1750
	SR	41	41
SR of pure outer membrane (A)		8	—70
SR of pure inner membrane (B)		209	270
A as % of B		3.8	—26

a densitogram of this preparation and also shows the distribution of radioactivity in the electrophoretic pattern. The specific radioactivities of the different bands, expressed in % of counts/min/% of amido black is strikingly similar in all bands (Fig. 3). It will be shown later that with rat-liver mitochondria the different bands can be coordinated to outer and inner membrane respectively. This indicates similarity of the labelling of outer- and inner-membrane proteins *in vivo*.

Finally, the necessity that non-membranous proteins are absent from the fractions used for calculation has already been discussed.

Electrophoresis of outer and inner membranes

As already mentioned, the insoluble mitochondrial membrane proteins can be separated into 20-25 bands on polyacrylamide gel in a medium containing phenol/formic acid/water (2 : 1 : 1). Fig. 4 represents such an electrophoresis of insoluble rat-liver

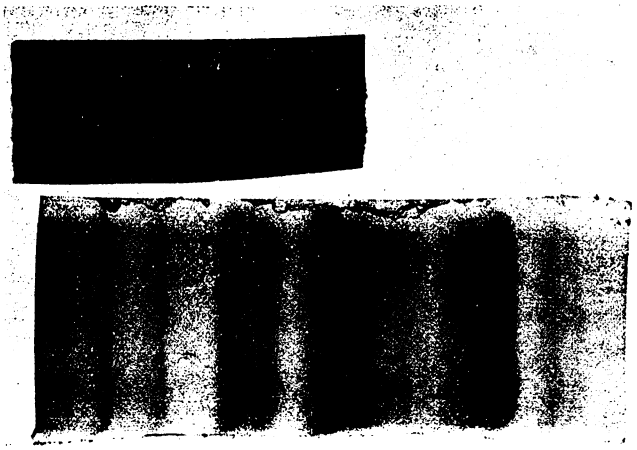

FIG 1 - Gel electrophoresis of insoluble mitochondrial protein from the flight muscle of *Locusta migratoria*. The insoluble protein is the sediment resulting from ultrasonic treatment in 0.1 M phosphate buffer (pH 7.3), followed by 30 min centrifugation at 40 000 rev./min in the Spinco 40 rotor. The electrophoresis was run for 5 h at 7 V/cm and the gel was stained with amido black; 0.2 mg of insoluble protein was applied.

mitochondrial protein. The distribution of the bands is strikingly similar to that of insoluble locust flight-muscle mitochondrial protein. Fig. 5 shows a densitogram of insoluble rat-liver mitochondrial protein and the distribution of radioactivity as it appears after the mitochondria were labelled *in vitro* with [^{14}C]-leucine, [^{14}C] isoleucine and [^{14}C] phenylalanine simultaneously. It can be seen that only four or five bands contain radioactivity. In Fig. 6 the middle pattern represents again the insoluble proteins of mitochondria before separation (fraction LM). Those four bands which contain radioactivity are marked. The pattern at the left represents the outer mitochondrial membrane preparation (fraction LI), the pattern at the right the inner mitochondrial membrane preparation (fraction LIII). It can be seen that those bands which are enriched in the outer membrane preparation are bands which do not contain radioactivity. On the other hand, in the inner membrane preparation bands are enriched containing radioactivity as well as bands not containing radioactivity. Interestingly, the degree of enrichment of the various bands in the inner membrane is not the same. It is therefore possible that the value of marker enzymes is limited and as

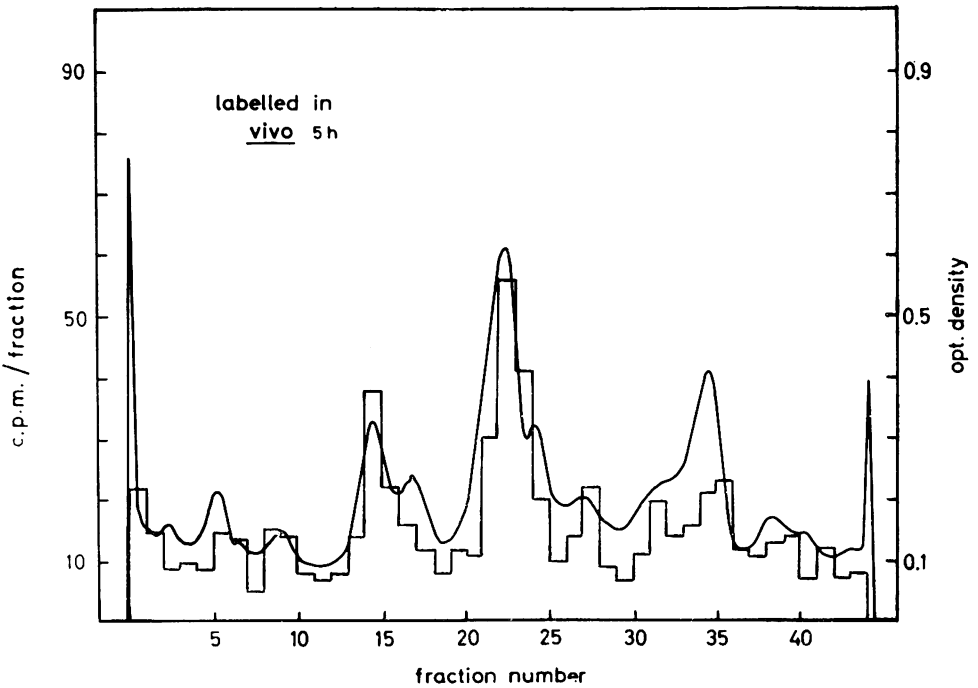

FIG. 2 - Amido black and [^{14}C] activity distribution in the electrophoretic pattern shown in Fig. 1. The mitochondria were labelled *in vivo* with [^{14}C] l-leucine. Continuous curve is amido black concentration.

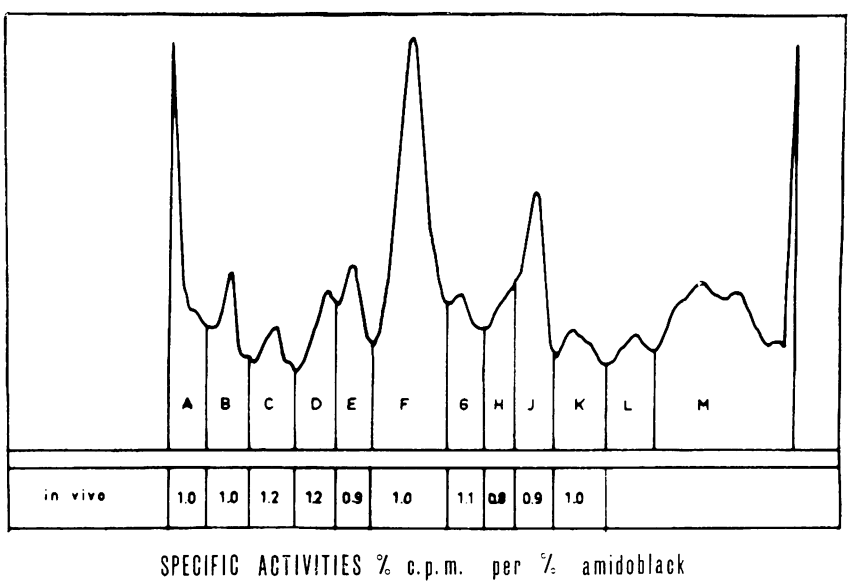

FIG. 3 - [^{14}C] Activity per amido black concentration in some fractions of the electrophoretic pattern shown in Figs. 1 and 2.

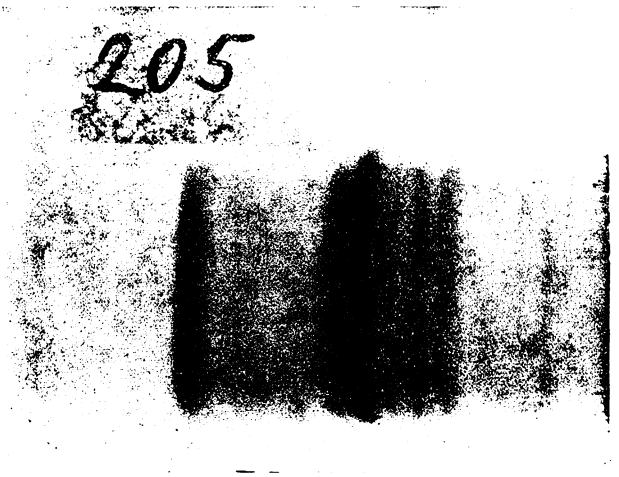

FIG. 4 - Gel electrophoresis of 0.2 mg insolub'e mitochondrial protein from rat liver. The electrophoresis was run for 7 h at 7V/cm.

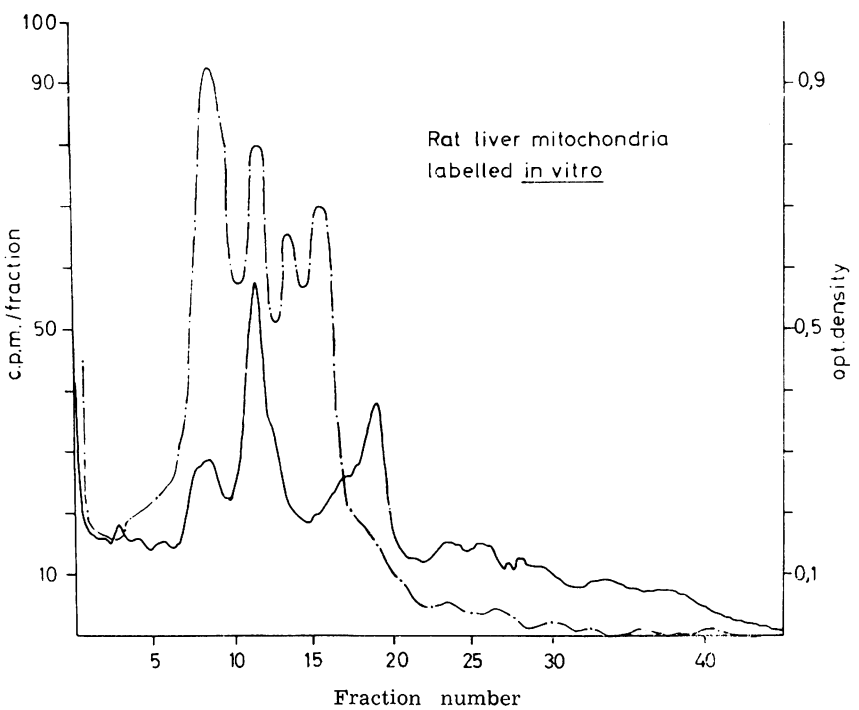

FIG. 5 - Distribution of amido black and [^{14}C] activity of insoluble mitochondrial protein after labelling *in vitro* with [^{14}C] l-leucine, [^{14}C] l-isoleucine and [^{14}C] l-phenylalanine. Broken curve is radioactivity.

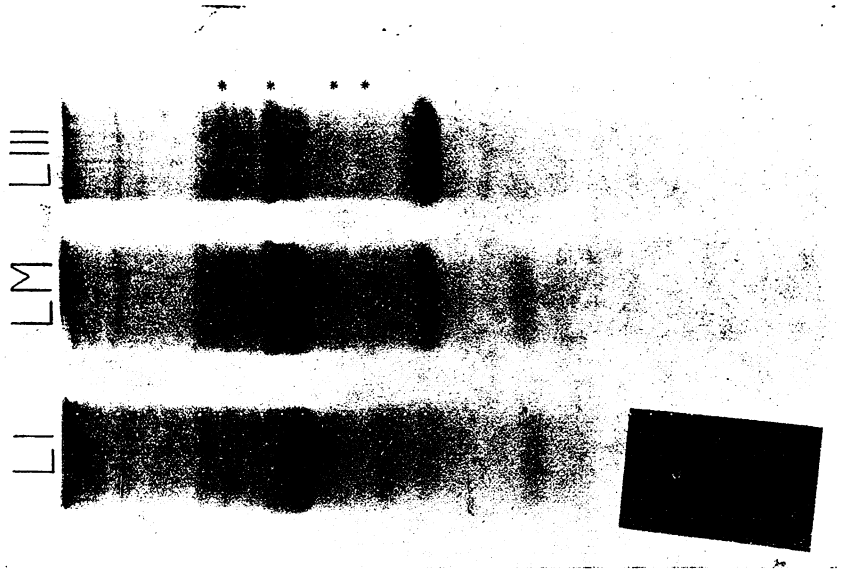

FIG. 6 - Electrophoretic patterns from gradient input (LM), enriched inner membrane (LI) and enriched outer membrane (LIII). All fractions were extracted in the manner described in Fig. 1. Stars correspond to the four bands containing radioactivity shown in Fig. 5.

already mentioned, the sites of incorporation and the marker enzymes may be located in different parts of the inner membrane. Because the single bands may consist of several different proteins, these electrophoretic experiments do not show the precise location of the newly synthesized proteins within the inner membrane. They do however, substantiate the results presented above.

Incubation of the different membrane preparations with radioactive leucine

Further information regarding the site of incorporation of amino acids into isolated mitochondria was obtained by incubating the membrane fractions prepared by the « swelling-shrinking » technique with [^{14}C] leucine. The results of two such experiments are shown in Table IV. Fraction LI has a low specific radioactivity when compared with fraction LII This means that the « pure » outer membrane is unable to incorporate radioactive leucine. The amino acid-incorporating

TABLE IV

Incorporation of [1-^{14}C] l-leucine into different fractions of mitochondria prepared by the « swelling-shrinking » method

The concentration of chloramphenicol used was 100 μg/ml, and that of RNAase 40 μg/ml. In Expt. 1, 0.4 and in Expt. 2, 0.2 μC/ml [^{14}C] l-leucine was used. Other conditions as described in text. Abbreviations: S, low-speed pellet (1900 x *g*); SII and SIII, bands corresponding to LII and LIII after S was subjected to density-gradient centrifugation.

Fraction	Addition	Specific radioactivity (counts/min per mg protein)	
		Expt. 1	Expt. 2
MS	None	114	330
	RNAase	116	
	Chloramphenicol	22	
LM	None	68	208
	Chloramphenicol	2	
S	None	252	1112
	RNAase	244	
	Chloramphenicol	12	
LI		17	44
LII	None	103	256
	Chloramphenicol	0	
LIII		98	108
SII		28	
SIII		74	
SR of pure outer membrane (A)		6	11
SR of pure inner membrane (B)		169	362
A as % of B		3.5	3.0

activity, on the other hand, appears to be bound to the inner membrane. However, the most active fraction is the low-speed pellet (fraction S). This fraction has a low monoamine oxidase activity indicating a low proportion of outer membrane. The capacity for incorporation of the S fraction is markedly lowered if it is centrifuged on the same gradient as the L fractions (SII and SIII). The incorporation into LII and S is sensitive to chloramphenicol and insensitive to ribonuclease. The properties of this subfractionated system are under further investigation.

The experiments described here present evidence to indicate that the outer mitochondrial membrane is not synthesized by the amino acid incorporating system of isolated rat-liver mitochondria. These findings might be relevant to the suggestion

of Parsons *et al.*[19] that the outer mitochondrial membrane is derived from the endoplasmic reticulum. They are consistent with the recent report of Clark-Walker and Linnane[23], who found that in glucose repressed yeast, chloramphenicol, which strongly inhibits amino acid incorporation into isolated mitochondria[17, 24], inhibits the formation of cristae but does not affect the formation of the outer mitochondrial membrane. Finally, our experiments with outer membrane-depleted mitochondria would strongly indicate that the site of protein synthesis is located within the inner mitochondrial membrane.

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