

The Significance of Promitochondrial Structures in Rat Liver for Mitochondrial Biogenesis

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1. The heavy, light and fluffy mitochondrial fractions obtained by differential centrifugation were further characterized with respect to their protein synthesizing ability *in vitro*, their nucleic acid content, buoyant density of their DNA and ultrastructure. 2. The light mitochondrial fraction synthesized proteins *in vitro* at a rate 4–5 times as high as heavy and fluffy mitochondria. The incorporation ability of this fraction was also maximally affected by the thyroid status of the animal. The radioactivity in leucyl-tRNA of the light mitochondrial fraction was about 3–4 times as high as that of the other two fractions. 3. The heavy, light and fluffy mitochondrial fractions contained small but consistent amounts of RNA and DNA. Although the DNA content was the same in all mitochondria fractions, the light mitochondria contained relatively more RNA. The buoyant density of DNA from all the fractions was 1.701 g/cm³. 4. Electron microscopy revealed that the heavy mitochondria have a typical mitochondrial architecture, with densely packed cristae and a well developed double membrane. Light mitochondria were also surrounded by double membranes, but were smaller in size and contained less cristae. The fluffy fraction consisted of a mixture of well formed mitochondria and those in the process of degradation. 5. The significance of these findings in relation to mammalian mitochondrial genesis is discussed.

The mode of mitochondrial genesis in mammalian cells is as yet unclear. The existence of mitochondrial heterogeneity and a study of the interrelationships among differentially separated fractions has provided a possible approach to this problem (Gear, 1965*a,b*). Previous observations from this laboratory have indicated that a rat liver mitochondrial population could be resolved into discrete heavy, light and fluffy entities having distinct differences in their properties (Katyare *et al.*, 1970). Our observations suggested that the light mitochondria could be immature forms in the process of developing into stable heavy mitochondrial structures by accretion of extra-mitochondrially synthesized soluble protein units; the fluffy particles appeared to be a mixture of regenerating and degenerating mitochondria.

In the present studies, an attempt has been made to characterize further the heavy, light and fluffy fractions with respect to their protein-synthesizing ability *in vitro* and their nucleic acids. Additionally, they have been characterized by electron-microscopic examination. The results support the postulate that the light mitochondrial fraction may represent transitional forms of the heavy mitochondria.

Materials and Methods

Chemicals

ATP, sodium succinate, L-amino acids, chloramphenicol, cycloheximide, 3,3',5-tri-iodo-L-thyron-

ine, 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyl-oxazol-2-yl)benzene, ribonuclease, deoxyribonuclease from bovine pancreas and crystalline bovine albumin were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. DL-[1-¹⁴C]Leucine (47.9 mCi/mmol) was obtained from the Isotope Division of this Research Centre. CsCl (optical grade) was obtained from Schwarz Bioresearch Inc., Orangeburg, N.Y., U.S.A. Epon 812 and methyl nadic anhydride were received from K & K Laboratories Inc., Plainview, N.Y., and Hollywood, Calif., U.S.A. *n*-Dodecyl succinic anhydride and 2,4,6-tri(dimethylaminomethyl)phenol (DMP-30) were obtained, respectively, from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and Rohm and Hass Co., Philadelphia, Pa., U.S.A. All other chemicals used were either AnalaR grade from BDH Chemicals Ltd., Poole, Dorset, U.K., or Pro Analyst quality from E. Merck A.-G., Darmstadt, Germany.

Micrococcus lysodeikticus was obtained from National Chemical Laboratory, Poona, India.

Animals and treatment with tri-iodothyronine

For studies involving control animals, male albino rats of the Wistar strain weighing 150–170 g were used. Thyroidectomy (Waynforth, 1969) was performed on male weanling rats of the same strain (30–35 g, 20–22 days old) by surgery and the animals were allowed to grow for 8–10 weeks, weight records

being maintained. Their basal metabolic rates were determined periodically in a closed-circuit apparatus as described by Maclagan & Sheahan (1950). Only those animals showing considerable decrease in body weight (30–35% decrease) and basal metabolic rate (35–40% decrease) were used for the studies. Triiodothyronine was dissolved in 0.9% NaCl at pH 8.5 by using KOH and was injected subcutaneously. Thyroidectomized animals received 20 µg of triiodothyronine/100g body wt. and the normal controls received 25 µg of triiodothyronine/100g body wt. 2 days before they were killed.

Preparation of rat liver mitochondrial fractions

The animals were killed by decapitation and livers were quickly removed and chilled in 0.25M-sucrose containing 10mM-Tris-HCl, pH 7.4, and 1mM-EDTA. The liver mitochondrial fractions (heavy, light and fluffy) were isolated as described previously (Katyare *et al.*, 1970) with certain modifications. Nuclear contamination in mitochondrial fractions was avoided by centrifuging the liver homogenate twice at 650g for 10min. The light mitochondrial fraction was sedimented at 12000g for 10min instead of 10000g for 10min. The fractions were washed three times by suspending them in 0.25M-sucrose solution containing Tris-HCl (10mM, pH 7.4) and EDTA (1mM) and resedimenting at the appropriate speeds. The resulting mitochondrial pellets were washed each time with small amounts of isolation medium by swirling. This, we observed, decreased the microsomal contamination to a considerable extent. The microsomal contamination was monitored in terms of the marker enzyme, glucose 6-phosphatase (Recknagel & Lombardi, 1961), and was always less than 3–5%.

Incorporation in vitro of DL-[1-¹⁴C]leucine

DL-[1-¹⁴C]Leucine incorporation was carried out *in vitro* in a medium described by Beattie *et al.* (1967), which contained 50mM-Tris-HCl, pH 7.2, 20mM-sodium phosphate buffer, pH 7.2, 10mM-sodium succinate, 5mM-MgCl₂, 2mM-EDTA, 2mM-ATP, 154mM-KCl, and 50 µg of complete amino acid mixture minus leucine (the final pH was adjusted to 7.2), 1.0 µCi of DL-[1-¹⁴C]leucine (sp. radioactivity 47.9mCi/mmol), 100 units of crystalline penicillin G and 3.0–5.0mg of mitochondrial protein in a total volume of 2.0ml. In inhibitor studies, 50 µg of D-chloramphenicol/ml or 500 µg of cycloheximide/ml was added to the incubation medium as indicated. The incubations were carried out in 50ml beakers in a Dubnoff metabolic shaker at 30°C with O₂ as the gas phase. At intervals of 30 and 60min, 0.4ml samples were removed and spotted in duplicate on Whatman 3MM paper strips (7cm × 1.8cm). One set

of strips was dried in a current of air and immediately dropped in a beaker containing cold 5% (w/v) trichloroacetic acid and 1mg of DL-leucine/ml. The strips were washed repeatedly with trichloroacetic acid and finally boiled in 5% trichloroacetic acid at 90°C for 30min to remove the radioactive amino acid charged on tRNA. The strips were then washed with trichloroacetic acid repeatedly, dried, extracted with ethanol-ether (1:1, v/v) followed by ether, dried and placed in vials containing 0.3% 2,5-diphenyl-oxazole and 0.01% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene as scintillator. Blank strips treated in a similar way showed near-background counts.

The other set of strips was given the usual treatments as described above except that the strips were not boiled in 5% trichloroacetic acid but were washed repeatedly with cold trichloroacetic acid. The radioactivity in these strips, we assumed, was due to incorporation into the protein as well as that associated with leucyl-tRNA (Fournier & Simpson, 1968). From the difference in the radioactivity between the two sets of strips, the radioactivity in the tRNA was calculated (Fournier & Simpson, 1968). The results of these studies were somewhat variable; however, the pattern was always reproducible.

Radioactivity was counted in a Beckman LS-100 liquid-scintillation spectrometer at 95% efficiency.

Protein determinations were carried out by the method of Lowry *et al.* (1951) with crystalline bovine albumin as a standard.

Determination of RNA and DNA

The mitochondrial fractions were first incubated with ribonuclease (Kroon, 1964) or deoxyribonuclease (Gross *et al.*, 1969), as appropriate, before extraction of nucleic acids by the procedure of Schmidt & Thanhauser (1945). RNA was determined by the method of Ceriotti (1955). DNA, after extraction in 0.5M-HClO₄, was determined by Burton's (1956) diphenylamine reaction.

Extraction of DNA for density-gradient analysis

The procedure followed for extraction of DNA from heavy, light and fluffy mitochondria was essentially that of Marmur (1961) except that, after deproteinization, extensive dialysis was carried out instead of precipitation of the nucleic acid by ethanol (Green *et al.*, 1967). Livers from 25 rats were pooled for the isolation of mitochondrial fractions for extraction of DNA. The mitochondrial fraction received a prior treatment with deoxyribonuclease, as described above, before extraction.

DNA from *Micrococcus lysodeikticus* was isolated by Marmur's (1961) procedure without modifications.

CsCl density-gradient centrifugation

Density-gradient centrifugation of DNA in CsCl was carried out as described by Schildkraut *et al.* (1962). A saturated solution of CsCl, prepared in 0.02M-Tris-HCl buffer, pH8.5, was filtered through sintered glass. A portion (0.83 ml) of this solution was then mixed with 0.23 ml of standard saline-citrate (0.015M-trisodium citrate in 0.15M-NaCl, pH7.0±0.2), containing 1-2µg of mitochondrial DNA plus 3-5µg of reference DNA from *M. lysodeikticus* ($\rho = 1.731\text{g/cm}^3$). The final density of the mixture was adjusted to 1.710g/cm³ with standard saline-citrate. The solution was then centrifuged at 44770rev./min, at 25°C for 22h in a standard 12mm Kel-F cell in the AN-D rotor of a Spinco model E analytical ultracentrifuge. Photographs were taken at 260nm and scanned with a Beckman Analytrol microdensitometer. Buoyant densities were calculated as described by Schildkraut *et al.* (1962).

Electron microscopy

The mitochondrial fractions were processed for electron microscopy by the procedure of Luft (1961). This method involves double fixation with 6.25% glutaraldehyde and 1% osmium tetroxide in phosphate buffer, 0.1M, pH7.4 (Millonig, 1961). Specimens were dehydrated by using graded concentrations of alcohol and embedded in Epon 812. Sections were prepared with a Porter-Blum MT-1 ultramicrotome, stained with uranyl acetate and lead citrate and examined in a Philips EM 200 electron microscope.

Results

Incorporation of DL-[1-¹⁴C]leucine in vitro

The results of incorporation of DL-[1-¹⁴C]leucine *in vitro* into the proteins of the three mitochondrial fractions of normal rat liver are shown in Fig. 1. The heavy and the fluffy mitochondrial fractions had almost identical ability to incorporate DL-[1-¹⁴C]leucine into their proteins *in vitro*, whereas the light mitochondrial fraction exhibited 4 to 5 times higher incorporation. The incorporation in all the fractions follows linear kinetics up to 1h.

Thyroid hormones greatly affect mitochondrial protein synthesis and turnover (Tata, 1964; Katyare *et al.*, 1970). Freeman *et al.* (1963) and Roodyn *et al.* (1965) demonstrated that administration of tri-iodothyronine to rats at physiological concentration stimulated incorporation of labelled amino acids into proteins by isolated liver mitochondria; they favoured the view that this treatment results in a selective synthesis of mitochondrial respiratory units. An increase in the number and size of skeletal muscle mitochondria on treatment with L-thyroxine has also been demonstrated (Gustafsson *et al.*, 1965).

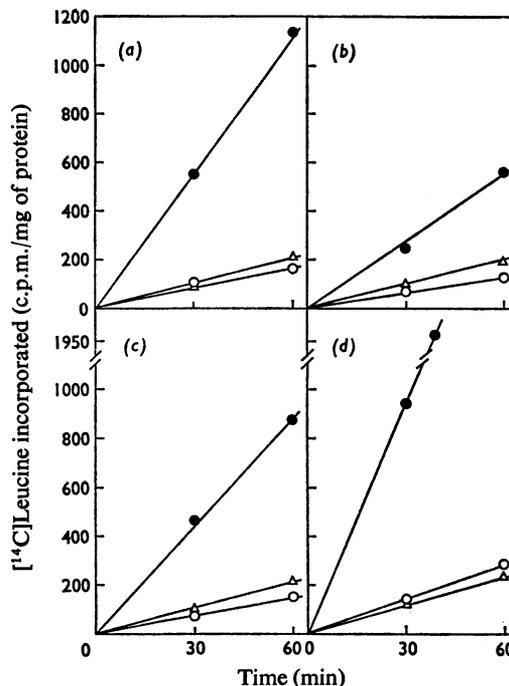


Fig. 1. Incorporation of DL-[1-¹⁴C]leucine by rat liver mitochondrial fractions *in vitro*

The incorporation system is described in the text. ○, Heavy, ●, light and Δ, fluffy mitochondrial fractions. (a) Normal rats; (b) thyroidectomized rats; (c) thyroidectomized rats given tri-iodothyronine; (d) normal rats given tri-iodothyronine.

In the light of these observations, we examined the effect of thyroid status of the animals on the incorporation of amino acid *in vitro* by these mitochondrial fractions with a view to magnifying the differences in their amino acid incorporation abilities. These studies were carried out with mitochondrial fractions isolated from livers of thyroidectomized, tri-iodothyronine-treated thyroidectomized and normal animals treated with tri-iodothyronine.

The results with the thyroidectomized animals are also shown in Fig. 1. It is evident that, compared with the normal (euthyroid) animals, the incorporation in the heavy fraction decreased by about 35%, whereas there was a decrease of 58% in the light mitochondrial fraction. The incorporation in the fluffy fraction, however, was almost unaffected under these conditions. It is noteworthy that, even under these conditions, i.e. in the thyroidectomized rats, incorporation in the light mitochondrial fraction was about 3-4 times as high as in the heavy fraction.

Treatment of the thyroidectomized rats with tri-iodothyronine (Fig. 1) resulted in a 20% stimulation

in the incorporation by the heavy fraction, but the incorporation in the fluffy fraction was almost unaffected under these conditions. However, there was a significant stimulation of 70% in the light fraction. Similar results were obtained for normal animals treated with tri-iodothyronine (Fig. 1), the stimulatory effect being more pronounced on the light mitochondrial fraction.

Radioactivity in tRNA of different mitochondrial fractions

In concomitant studies, the radioactivity in leucyl-tRNA of the different mitochondrial fractions was also examined. The results are shown in Table 1. It is evident that, although the radioactivity in tRNA expressed as c.p.m./mg of protein is comparable for heavy and fluffy fractions, the light mitochondrial fraction shows about 3–4 times higher specific radioactivity. The radioactivity at 30 and 60 min was almost identical or increased slightly at 60 min. Similar results were obtained with thyroidectomized, tri-iodothyronine-treated thyroidectomized, and normal animals treated with tri-iodothyronine (Table 1).

Dependence of DL-[1-¹⁴C]leucine incorporation in vitro on substrate and ATP

Our previous studies had indicated that, in contrast with the heavy mitochondria, the light mitochondrial fraction is unable to carry out coupled phosphorylation, but can oxidize substrates such as succinate (Katyare *et al.*, 1970). In view of this

observation, we decided to examine the dependence of DL-[1-¹⁴C]leucine incorporation *in vitro* on ATP and succinate. Such incorporation studies were carried out with heavy, light and fluffy mitochondrial fractions (Table 2), and show that the incorporation is independent of exogenously added ATP and succinate.

Effect of site-specific inhibitors

The results in Table 3 show the effects of chloramphenicol (50 µg/ml) and of cycloheximide (500 µg/ml) on incorporation by heavy, light and fluffy mitochondrial fractions. The incorporation in all the mitochondrial fractions was inhibited equally to the extent of 52–54% by chloramphenicol, whereas cycloheximide, a known inhibitor of microsomal protein synthesis (Borst *et al.*, 1967), did not have any significant effect. The results thus indicate that the observed incorporation is truly mitochondrial.

Nucleic acid contents

Table 4 shows that the heavy, as well as fluffy fractions, do not differ significantly in their contents of RNA/mg of protein, whereas the light mitochondrial fraction has a higher content. This is reflected in the higher protein-synthesizing ability *in vitro* observed in this fraction (Fig. 1). The values for DNA content show that the three mitochondrial fractions possess small but almost equal amounts of DNA/mg of protein. In other experiments, we observed that the thyroid status of the animal does not

Table 1. Radioactivity in leucyl-tRNA of mitochondrial fractions

Incubated mitochondrial samples were spotted on Whatman 3MM paper strips in duplicate. One set of strips was washed with hot trichloroacetic acid. The other set was washed with cold trichloroacetic acid. From the difference, the radioactivity in leucyl-tRNA was calculated. The details are described in the text. The results are typical of five or six independent experiments.

State of the animals	Mitochondrial fraction	Sp. radioactivity (c.p.m./mg of protein)	
		30min	60min
Normal	Heavy	114.3	125.2
	Light	303.9	480.8
	Fluffy	65.9	81.0
Thyroidectomized	Heavy	147.6	137.3
	Light	396.6	448.1
	Fluffy	179.6	96.1
Thyroidectomized + tri-iodothyronine (48h)	Heavy	121.3	124.7
	Light	406.4	429.5
	Fluffy	173.4	150.5
Normal + tri-iodothyronine (48h)	Heavy	101.4	103.4
	Light	302.3	438.0
	Fluffy	155.8	178.4

Table 2. ATP and substrate requirement for incorporation in vitro of DL-[1-¹⁴C]leucine by mitochondrial fractions

The incorporation system is described in the text. ATP, succinate, or both were omitted from the system as indicated. Incubation was carried out for 1h. The results are typical of five sets of experiments.

Mitochondrial fraction	Sp. radioactivity (c.p.m./mg of protein)			
	+ATP	+ATP	-ATP	-ATP
	+Succinate	-Succinate	+Succinate	-Succinate
Heavy	358	375	332	304
Light	1042	994	874	1031
Fluffy	328	360	332	308

Table 3. Effect of chloramphenicol and cycloheximide on DL-[1-¹⁴C]leucine incorporation in vitro by mitochondrial fractions

The incorporation system is described in the text. Chloramphenicol (50 µg/ml) and cycloheximide (500 µg/ml) were added as indicated. The incubation was carried out for 30min. The results are typical of four sets of experiments.

Mitochondrial fraction	Sp. radioactivity				
	Control (c.p.m./mg of protein)	+Chloramphenicol		+Cycloheximide	
		(c.p.m./mg of protein)	Inhibition (%)	(c.p.m./mg of protein)	Inhibition (%)
Heavy	87.8	39.1	52.8	74.0	10.8
Light	783.6	358.0	54.3	774.2	1.2
Fluffy	90.6	48.0	53.0	88.6	2.2

Table 4. Nucleic acid content of mitochondrial fractions

The nucleic acids were extracted by Schmidt & Thanhauser's (1945) method. RNA and DNA were determined, respectively, by Ceriotti's (1955) and Burton's (1956) procedures as described in the text. Results are mean of five independent determinations ± S.E.M.

Mitochondrial fraction	Content (µg/mg of protein)	
	RNA	DNA
Heavy	6.83 ± 0.60	0.165 ± 0.016
Light	20.99 ± 1.38	0.168 ± 0.012
Fluffy	5.78 ± 0.44	0.176 ± 0.006

affect the specific content of either RNA or DNA of these mitochondrial fractions (M. S. Rajwade, S. S. Katyare, P. Fatterpaker & A. Sreenivasan, unpublished work). Thus, the RNA contents, expressed as µg/mg of protein, of the heavy, light and fluffy mitochondria of the thyroidectomized animals were

respectively 6.4, 20.0 and 6.0; corresponding values for DNA were 0.167, 0.166 and 0.170.

Buoyant density of mitochondrial DNA in CsCl

Fig. 2 shows the densitometric tracings of the DNA obtained from the heavy, light and fluffy fractions. The DNA samples from all the three fractions have identical buoyant densities of 1.701 g/cm³, compared with the known value of 1.703 g/cm³ for rat liver nuclear DNA (Schneider & Kuff, 1965). The results thus prove that the DNA is of mitochondrial origin. Absence of any other peak in these fractions also rules out the possibility of contaminants, besides confirming that the three fractions are distinct entities and not artifacts of preparation.

Electron microscopy

When examined under the electron microscope, the heavy mitochondria appeared to be well organized structures with densely packed cristae and intact double membranes. The light mitochondria were much smaller in size than their heavy counterparts, with less cristae space, as expected, if we assume that

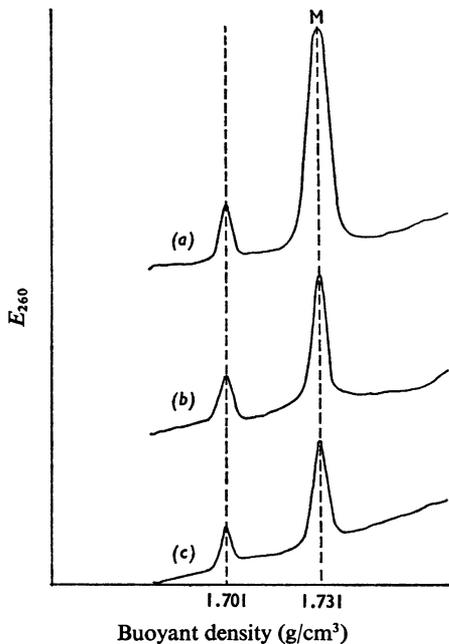


Fig. 2. Microdensitometer tracings of absorption photographs of DNA from (a) heavy, (b) light and (c) fluffy mitochondrial fractions at equilibrium in a $CsCl$ gradient

Details are as given in the Materials and Methods section. M, *Micrococcus lysodeikticus* marker DNA ($\rho = 1.731 \text{ g/cm}^3$).

they represent young immature mitochondria (Katyare *et al.*, 1970). Various stages of maturation could also be seen in the light fraction. These ranged from vacuolar structures surrounded by double membranes, in which the cristae were totally absent, to the rudimentary mitochondria with partially formed cristae, and to the relatively more mature forms with typical mitochondrial architecture. However, in this latter population also, the cristae space was much less than that in the fully developed heavy mitochondria, and they therefore appeared vacuolated. Such an appearance is typical also of mitochondria in early embryonic tissues of mouse (Calarco & Brown, 1968) and rat (Mackler *et al.*, 1971); a marked increase in the development of cristae is seen as the embryo develops. The fluffy fraction, on the other hand, represents a mixture of heavy and degenerating mitochondria. Frequently, areas were seen where mitochondrial structures were completely disorganized with loss of outer membrane and with inner contents oozing out. Possibly the structures, which apparently looked intact like heavy mitochondria, might also have undergone functional im-

pairment, not reflected as yet in structural alterations, as is evident from a low ADP/O ratio and a high Mg^{2+} -activated adenosine triphosphatase activity in this fraction (Katyare *et al.*, 1970).

Discussion

The present studies imply that the three mitochondrial fractions behave differentially with respect to their ability to carry out incorporation *in vitro* of DL-[1- ^{14}C]leucine into their proteins. Also, the light mitochondrial fraction shows most significant changes as a response to the thyroid status of the animals. The differential pattern of label incorporation into the proteins of heavy, light and fluffy mitochondria would remain essentially unaltered even when expressed per mg of mitochondrial DNA, since the specific DNA content is nearly the same in all the three fractions (Table 4).

The mitochondrial fractions used in these studies were washed at least three times and microsomal contamination was monitored in terms of a microsomal marker enzyme, glucose 6-phosphatase; assessed in this way, the percentage contaminations in the heavy, light and fluffy fractions were, 3, 5 and 5, respectively. Since glucose 6-phosphatase is known to be a constitutive enzyme of the outer membrane of the mitochondria (Brunner & Bygrave, 1969), the actual microsomal contamination may be much less than the observed value. The contribution, by contaminating microsomal fractions to the incorporation is therefore minimum. It is also known that the microsomal preparation does not incorporate amino acids to a significant extent under these conditions of incorporation (Wheeldon & Lehninger, 1966). The studies with cycloheximide and chloramphenicol (Table 3) also favour true mitochondrial protein synthesis *in vitro*.

The possibility of bacterial contamination is ruled out for the following reasons. (1) The incubations were carried out in the presence of penicillin G, which is known to kill most of the Gram-positive bacteria. (2) Bacterial colony counting, by plating the incubated mitochondria at the end of incubation, revealed less than 5×10^4 colonies/ml; this may be aerial contamination. Such a bacterial contamination does not significantly contribute to mitochondrial protein synthesis (Kroon *et al.*, 1968). (3) The effect of thyroid status of the animals on the incorporation ability of the different mitochondrial fractions is quite obvious and cannot be explained on the basis of bacterial and/or microsomal contamination. The high synthetic ability of the light mitochondrial fraction *in vitro* therefore appears to be a true property of this fraction.

The results on increased amounts of radioactivity in leucyl-tRNA of the light fraction (Table 1) together with the observations on its elevated RNA con-

tent (Table 4) appear to be in agreement with its high synthetic activity *in vitro*. Similar results *in vivo* have been reported (Katyare *et al.*, 1970). It is interesting to see that the protein synthetic activity of the three classes of mitochondria does not respond in the same manner to a hormonal stimulus. This functional heterogeneity of the mitochondrial fractions is in turn reflected in their recoveries; the proteins of the light mitochondrial fraction decrease by 40% on thyroidectomy and increase by 160% on tri-iodothyronine administration, whereas the changes in specific protein contents of the other two fractions taken together are not significant (Katyare *et al.*, 1970). Since it was difficult to isolate the fluffy particles from the heavy fraction quantitatively, these two fractions were pooled in the above study.

Although thyroidectomy resulted in a decrease in the protein synthesis and subsequent treatment with tri-iodothyronine brought about an increase in synthetic activity of mitochondria in general, the radioactivity in the tRNA (Table 1) did not change to a significant extent. Also, no changes in specific RNA contents of the heavy, light and fluffy fractions were observed with respect to thyroid status of the animals. The effect of thyroid hormones, therefore seems to be related to the transfer step, rather than to the activation of amino acid during mitochondrial protein synthesis *in vitro*.

It is noteworthy that the incorporation *in vitro* in the heavy as well as the light mitochondrial fraction is not dependent on exogenously added succinate and/or ATP. This would mean that probably these fractions contain enough endogenous substrates, or that high-energy intermediates other than ATP may be involved in the activation step. Such an observation is also supported by the findings on the resistance towards arsenate of amino acid incorporation by isolated mitochondria (Ozawa *et al.*, 1970). However, the possibility of amino acids being deaminated and utilized in the tricarboxylic acid cycle as suggested by Wheeldon & Lehninger (1966) cannot be ruled out. The observation of Kováč (1972) that, in aerobically growing yeast cells, formation of mitochondria is not obligatorily dependent upon energy generated by the organelle, is of relevance in this connexion.

According to the present concept of mitochondrial biogenesis, mitochondrial proteins arise by dual routes. Soluble proteins such as cytochrome *c* are synthesized on cytoplasmic ribosomes and insoluble membrane proteins are probably synthesized by mitochondrial ribosomes (Gonzalez-Cadavid & Campbell, 1967; Freeman *et al.*, 1967; Beattie *et al.*, 1966; Kadenbach, 1967*a,b*). Co-ordination of the two systems and integration of the proteins synthesized by the two routes eventually leads to the formation of mitochondria (Work *et al.*, 1968; Roodyn & Wilkie, 1968). Synthesis of insoluble membrane proteins is considered to be a prerequisite

for the incorporation of soluble proteins (Beattie, 1968). It is therefore reasonable to expect that the immature mitochondrial structures would possess ability for incorporation *in vitro*. Promitochondria of anaerobically grown yeast contain a specific amount of DNA and are also capable of protein synthesis *in vitro* (Criddle & Schatz, 1969; Plattner & Schatz, 1969). The results of the present studies show that the light mitochondrial fraction possesses a high synthetic ability *in vitro* that is maximally affected by the thyroid status of the animal. The report (Hallman, 1971) that a fraction derived from light mitochondria is the site of active protein synthesis in mitochondrial membranes lends significance to these findings.

In other studies, we have also observed that, after injection of [³H]glycerol *in vivo*, highest radioactivity is found in the phospholipids of the light fraction, particularly in the cardiolipin component (J. G. Satav, S. S. Katyare, P. Fatterpaker & A. Sreenivasan, unpublished work). These observations again support the promitochondrial nature of this fraction.

A small but reproducible amount of DNA is always found associated with these mitochondrial fractions. This DNA appears to be truly mitochondrial, because the mitochondrial fractions were prepared taking care to avoid non-mitochondrial contamination, and because the fractions so obtained were given deoxyribonuclease treatment before extraction of DNA. Also, it was ascertained in separate experiments that 18h after intraperitoneal injection of [³H]thymidine, incorporation of label in the DNA of the heavy, light and fluffy fractions was much higher (228–235 d.p.m./ μ g of DNA) compared with relatively negligible radioactivity (28 d.p.m./ μ g of DNA) in the nuclear fraction (M. S. Rajwade, S. S. Katyare, P. Fatterpaker & A. Sreenivasan, unpublished work). Further proof for the mitochondrial specificity of this DNA comes from our observations on CsCl density-gradient centrifugation. The DNA samples from heavy, light and fluffy mitochondrial fractions all form a band in CsCl at 1.701 g/cm³ without detectable nuclear or bacterial contamination. These observations, besides emphasizing the purity of these fractions, strongly suggest autonomy and physical continuity among the three mitochondrial fractions which represent the young, the mature and the degrading forms of the same structure.

Previously, we observed that the light mitochondrial fraction has a rapid turnover and a high synthetic ability *in vivo* (Katyare *et al.*, 1970). This fraction also accreted preformed soluble protein units under the influence of thyroid hormones. These results, taken together with the present findings, would support our earlier postulate that the light mitochondria represent transitional structures that eventually grow to form heavy mitochondria. The fluffy fraction behaves somewhat similarly to the

heavy, probably because it contains a mixture of regenerating and degenerating mitochondria. Indeed, there is increasing evidence of the presence of more than one type of mitochondria in mammalian tissues. Of particular interest in this context are the observations of Pollak & Munn (1970), Mackler *et al.* (1971), Ch'ih & Delvin (1971), Yago *et al.* (1972) and Bondi *et al.* (1972) on two different types of mitochondrial population in different animal tissues consisting of newly formed and mature mitochondria, the proportions of which vary with physiological conditions. The findings of Wilson & Cascarano (1972) also indicated biochemical heterogeneity of a rat liver mitochondrial population separated by rate zonal centrifugation in respect of cytochromes, glycerol phosphate dehydrogenase and succinate dehydrogenase. The observations of Criddle & Schatz (1969) and Plattner & Schatz (1969) on the promitochondria of anaerobically growing yeast are also of considerable relevance. These organelles, besides possessing oligomycin-insensitive adenosine triphosphatase, similar to the dinitrophenol-insensitive adenosine triphosphatase of our light mitochondria, also possess specific amounts of DNA and RNA as well as ability to incorporate labelled amino acids into their proteins *in vitro*. Further, Plattner *et al.* (1970) showed the transformation of the pre-labelled promitochondria into fully grown mitochondria. Under these conditions, the label was quantitatively associated with the mitochondria when the yeast cells were subjected to aeration. Such studies are difficult in animal systems, since it is not possible to have exclusively either the promitochondria or the fully grown mitochondria. The properties of the different mitochondrial fractions, however, can be manipulated with respect to thyroid status as in the present studies.

A more direct proof for the existence of mitochondrial heterogeneity has come from our electron-microscopic observation on the three distinct types of mitochondrial population in rat liver. It appears that the light mitochondrial fraction contains promitochondria-like bodies at various stages of maturation. The fluffy fraction depicts the phase of destruction of the mature heavy mitochondria. It should be pointed out here that the mitochondrial fractions were isolated by differential centrifugation and were not subjected to sucrose density gradients; the possibility of mitochondrial structure being damaged owing to the stress of the gradient centrifugation (Wattiaux *et al.*, 1971) is therefore ruled out. Ultrastructural degradation of mitochondria in sucrose-density gradients is reported by several workers (Simon *et al.*, 1969; Sacktor & Shimada, 1972).

Although the present studies reveal that the three mitochondrial fractions have different structures and properties, with the light mitochondria being prob-

ably the young premature forms, they do not indicate the mode of formation of the light mitochondria. If it is assumed that they are formed by growth and division of the heavy mitochondria, then it is reasonable to expect that the newly formed mitochondria would have properties identical with those of the parent mitochondria. However, according to such an assumption (Droz & Bergerson, 1965), the lack of oxidative phosphorylation in light mitochondria (Katyare *et al.*, 1970), which is probably due to deficiency of coupling factor(s), and their response to the treatment with thyroid hormones, remain unexplained. More work needs to be done to elucidate these and other aspects. Nevertheless, the present observations only emphasize the complexity of events that result in the assembly of a mitochondrion (Schatz, 1970).

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