Biochem. J. (1970) **118**, 111–121
Printed in Great Britain

Heterogeneity of Rat Liver Mitochondrial Fractions and the Effect of Tri-Iodothyronine on their Protein Turnover

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(*Received* 1 *December* 1969)

1. Rat liver mitochondria were separated into heavy, light and fluffy fractions by differential centrifugation under standard conditions. 2. All mitochondrial fractions possessed soluble as well as membrane-bound enzymes typical of mitochondria. 3. The heavy fraction represented the stable mitochondrial structures and the fluffy particles appear to be loosely coupled. 4. The light mitochondrial fraction lacked the ability of coupled phosphorylation. 5. A study of mobility and isoelectric pH indicated a similarity in the basic membrane structure of all the mitochondrial fractions. 6. The turnover rates of proteins in the heavy and fluffy particles were almost identical; however, this rate was rapid for the light mitochondrial fraction. 7. On treatment with 3,3′,5-tri-iodo-L-thyronine, succinoxidase activity was maximally stimulated much earlier in the light mitochondrial fraction than in the heavy fraction. The activity of the fluffy particles, however, remained almost unaffected. 8. Malate dehydrogenase activity in all the mitochondrial fractions was stimulated only at 40h after tri-iodothyronine treatment. 9. The pattern of incorporation of DL-[1-^{14}C]leucine *in vivo* in the tri-iodothyronine-treated animals indicated a rapid initial incorporation and high synthetic ability of the light mitochondrial fraction. 10. The turnover pattern of proteins of the mitochondrial fractions from animals receiving repeated doses of tri-iodothyronine was remarkably different from the normal pattern and suggested that preformed soluble protein units may be incorporated in the light mitochondrial fraction during maturation to form the stable heavy mitochondria. 11. The amount of light-mitochondrial proteins decreased by 40% on thyroidectomy and increased by 160% on treatment with tri-iodothyronine. 12. The possible significance of these results is discussed in relation to mitochondrial genesis.

Mitochondrial heterogeneity with respect to sedimentation characteristics, chemical make-up and enzymic activities has long been a matter of considerable discussion. Kuff & Schneider (1954) showed some degree of mitochondrial heterogeneity with respect to succinate dehydrogenase. Similarly Frisell, Patwardhan & Mackenzie (1965) isolated heavy and light fractions of mitochondria and showed that the heavy fraction had twice as much sarcosine dehydrogenase activity as the light fraction, although no differences could be found in four other enzymes studied. Gear (1965*a*,*b*), working with regenerating rat liver, separated mitochondria into heavy, light and fluffy fractions and concluded from his studies that the light fraction may have a precursor role whereas the fluffy particles may represent a mixture of regenerating and degenerating mitochondria with microsomes. Heterogeneity has also been observed in yeast mitochondria (Matile & Bahr, 1968). On the other hand, de Duve and co-workers strongly favour the view that the mitochondrial population is not heterogeneous and ascribe the observed differences to abnormalities caused by sucrose concentration and contamination of mitochondria by other cell organelles or fragmentation during cell homogenization (Beaufay *et al.* 1964; de Duve, 1967).

Since mitochondria have a fairly short half-life of about 8.5 days (Fletcher & Sanadi, 1961; Beattie, Basford & Koritz, 1967), the mitochondrial population may be expected to vary with respect to age and chemical make-up, which would be reflected in enzymic activities. The findings of Gear (1965*a*,*b*) are consistent with this view but are not yet fully substantiated.

Our interest in mitochondrial heterogeneity arose from the fact that the mitochondrial population obtained by differential centrifugation and consisting of heavy, light and fluffy fractions

exhibits differences in respect of their stability and certain membrane-bound enzyme activities in a stress condition caused by administration of carbon tetrachloride (Fatterpaker, Bhuvaneswaran, Patwardhan & Sreenivasan, 1965).

In the present studies, an attempt has been made to distinguish and differentiate the heavy, light and fluffy mitochondrial fractions with respect to membrane-bound and soluble enzymes, capacity to carry out oxidative phosphorylation, stimulation of ATPase* by Mg^{2+} and 2,4-dinitrophenol and electrophoretic mobility. An attempt has also been made to study the turnover rates of these organelles with a view to determining their half-lives.

Since the thyroid hormones, thyroxine and 3,3',5-tri-iodo-L-thyronine, have been shown to have a pronounced effect in regulating the metabolism of mitochondria and could cause selective synthesis of respiratory units in mitochondria (Freeman, Roodyn & Tata, 1963; Gustafsson, Tata, Lindberg & Ernster, 1965; Roodyn, Freeman & Tata, 1965; Tata *et al.* 1963), treatment of thyroidectomized animals with tri-iodothyronine has been used to magnify the differential behaviour of the mitochondrial fractions and to illustrate, from studies on protein synthesis and turnover rates, the relationship in these particles.

MATERIALS AND METHODS

Chemicals. All reagents used were of A.R. grade. Succinic acid was a product of E. Merck. A.-G., Darmstadt, Germany. NAD^+, NADH, cytochrome *c* (type II), malic acid, oxaloacetic acid, ADP, ATP and glucose 6-phosphate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. DL-β-Hydroxybutyric acid was obtained as the sodium salt from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Malic acid and oxaloacetic acid were used as sodium salts at pH 6.8. 2,4-Dinitrophenol was a product of British Drug Houses Ltd., Poole, Dorset, U.K., and was recrystallized before use. 2,4-Bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen was purchased from Packard Instruments Co. Inc., La Grange, Ill., U.S.A. DL-[1-^{14}C]-Leucine (52.3 mCi/mmol) was obtained from the Isotope Division of this Research Centre.

A gift of 3,3',5-tri-iodo-L-thyronine from Glaxo Laboratories Ltd., Greenford, Middx, U.K., is gratefully acknowledged.

Animals and treatment with tri-iodothyronine. In studies with mitochondrial fractions of normal rat liver, albino rats of Wistar strain (150–170 g, male or female) were used. In studies involving use of tri-iodothyronine, thyroidectomy was carried out on weanling rats weighing about 50–60 g (20–22 days old) by surgery. The animals were allowed to grow for 8–10 weeks and then used for the subsequent studies.

The animals were weighed and basal metabolic rates were determined periodically as described by Maclagan & Sheahan (1950). Rats showing considerable decrease in body weight (30–35%) and basal metabolic rate (35–40%) were only used for tri-iodothyronine administration and further studies. Basal metabolic rates of animals receiving tri-iodothyronine were determined within 1 h before they were killed. Tri-iodothyronine was dissolved in 0.9% NaCl adjusted to pH 8.5 with KOH solution and was injected subcutaneously. Both acute and chronic treatments were used. In acute treatment the animals received 20 μg of tri-iodothyronine/100 g body wt. and during chronic treatment the animals received 20 μg of tri-iodothyronine/100 g body wt. on the first day and then 15, 10, 10 and 10 μg of tri-iodothyronine/100 g body wt. on subsequent alternate days. Such repeated doses of tri-iodothyronine were not toxic to the animals and sustained the stimulation of protein synthesis during the experimental period (Tata *et al.* 1963).

During the study of enzyme profiles with respect to tri-iodothyronine treatment, the animals received a single dose of tri-iodothyronine as described above and were killed at intervals of 3, 10, 18, 24, 40, 48, 65 and 90 h.

Preparation of subcellular fractions. Rats were decapitated and the livers were quickly removed and chilled in 0.25 M-sucrose. A 10% liver homogenate was made at 0–4°C in 0.25 M-sucrose in a Potter–Elvehjem-type homogenizer with a Teflon pestle, giving two strokes at a low speed of 1000–1200 rev./min.

The isolation of liver mitochondrial fractions was carried out as described by Werkheiser & Bartley (1957) with some modifications. The nuclei were removed by centrifuging the homogenate at 650*g* for 10 min. The supernatant was centrifuged at 6500*g* for 10 min to sediment the crude heavy mitochondrial fraction. The clear supernatant was carefully decanted and centrifuged at 10000*g* for 10 min to give the light mitochondrial fraction. The crude heavy mitochondrial pellet obtained at 6500*g* consisted of two populations: a firmly packed brownish layer of heavy mitochondria and a loosely sedimenting pinkish fluffy layer on the top. These fluffy particles were gently poured off, resedimented for 10 min at 6500*g* from 0.25 M-sucrose and designated the 'fluffy' fraction. The technique for pouring out the fluffy particles was standardized so as to maintain uniformity in the procedure throughout. The residual fluffy particles from the heavy fraction were sloughed off by layering a small amount of sucrose on the sediment and giving a swirling movement. This procedure was repeated until no more fluffy particles could be separated. These washings were discarded since they contained a considerable amount of heavy mitochondrial fraction. All mitochondrial fractions were washed once by suspending in 0.25 M-sucrose and resedimenting at the respective speeds. The mitochondria-free supernatant was centrifuged at 105000*g* for 60 min to sediment the microsomes. The centrifugations were carried out at 0–4°C in an International Refrigerated PR-2 centrifuge or an MSE Superspeed 40 centrifuge.

It was ascertained that the three mitochondrial fractions, heavy, light and fluffy, obtained by the procedure outlined above have definable properties in terms of enzyme activities, protein turnover and electrophoretic mobilities (results reported below) that could be reproduced with a variation of ±8–10%, thus pointing to their probable occurrence as discrete entities.

For quantitative isolation of mitochondrial fractions, care was taken not to lose any fraction either in the

* Abbreviation: ATPase, adenosine triphosphatase.

nuclear sediment or during washing. Since it was difficult to isolate the fluffy particles from the heavy fraction quantitatively, these two fractions were pooled in this study. The protein values were corrected for microsomal contamination. The values are expressed as percentages of the control.

Assay of enzyme activity. Succinoxidase activity in the mitochondrial fractions was assayed by the procedure described by Potter (1959) and malate dehydrogenase activity was measured by the method of Ochoa (1955). NADH–cytochrome *c* reductase activity was measured by the procedure of Corwin & Schwarz (1959), D-β-hydroxybutyrate dehydrogenase by the method of Lehninger, Sudduth & Wise (1960) and glucose 6-phosphatase by the method of Recknagel & Lombardi (1961). ADP/O ratio and respiratory control index were determined in a Gilson model KM Oxygraph (Gilson Medical Electronics, Middleton, Wis., U.S.A.) by the method described by Chance & Williams (1955, 1956). Stimulation of ATPase in the presence and absence of Mg^{2+} and/or 2,4-dinitrophenol was measured by the method of Veldsema-Currie & Slater (1968).

The P_i liberated in the assay of glucose 6-phosphatase and ATPase was determined by the method of Fiske & Subbarow (1925).

Electrophoretic mobility of mitochondrial fractions. Mobilities of mitochondria in 0.25M-sucrose were studied in a cell-electrophoresis apparatus as described by Bangham, Flemans, Heard & Seaman (1958). The time taken to traverse a certain number of squares of the graticule in the eye-piece was observed by using a stopwatch measuring down to 10ms. The process was repeated for the same particle by reversing the direction of applied electric field. Transit time for 15–20 mitochondrial particles were recorded for each independent experiment. The dependence of mobility on pH was studied in the veronal–sodium acetate–HCl buffer of Michaelis (1931) except that KCl was replaced by 0.25M-sucrose. The pH–mobility curves were plotted as described by Boulter, Douglas & Laycock (1965).

Injection of DL-[1-^{14}C]*leucine.* Control rats weighing about 150–160g received 10μCi of DL-[1-^{14}C]leucine/100g body wt. intraperitoneally and were killed after 2, 4 or 10 days.

For incorporation studies the animals received intraperitoneal injections of DL-[1-^{14}C]leucine (15μCi/100g body wt.) at 30, 45 and 70h after tri-iodothyronine administration and were killed 2h thereafter.

For turnover studies, the animals received DL-[1-^{14}C]-leucine (15μCi/100g body wt.) intraperitoneally 2 days after the first dose of tri-iodothyronine. The animals were then given tri-iodothyronine subcutaneously on alternate days as indicated above. The animals were killed at 2, 4, 6, 8 or 10 days after the intraperitoneal injection of DL-[1-^{14}C]leucine.

Mitochondrial fractions were washed four times to remove the extraneous radioactivity.

Assay of radioactivity. Radioactivity was counted in a liquid-scintillation spectrometer by the method of Mans & Novelli (1961) as modified by Roodyn *et al.* (1965). Of the mitochondrial suspension, 0.2ml was spotted on Whatman no. 3MM filter-paper strips (7cm × 1.8cm). After spots of the mitochondrial suspension had been applied, the strips were dried with a current of air, further processed by the method of Roodyn *et al.* (1965) and their radioactivities counted in a Packard model 314 EX liquid-scintillation spectrometer with 0.4% 2,5,bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen in toluene as a scintillator. The counting efficiency as judged by counting the radioactivity of the standard was 68%. The orientation of the paper strips in the bottle appeared to have no effect on counting efficiency.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

RESULTS

Contamination of mitochondria with microsomes

The mitochondrial fractions were found to be contaminated by microsomes to various degrees. Correction for microsomal contamination was therefore applied by determining glucose 6-phosphatase, a typical microsomal enzyme, as a marker (Table 1).

Properties of mitochondrial fractions obtained from normal rat liver

Respiratory enzymes of mitochondrial fractions. The succinoxidase activity is somewhat less in the light mitochondrial fraction and is higher in fluffy particles than in the heavy mitochondrial fraction (Table 2). Addition of cytochrome *c* resulted in stimulation of the activity in all the fractions, the

Table 1. *Contamination of mitochondrial fractions with microsomes*

The glucose 6-phosphatase activity was determined by the method of Recknagel & Lombardi (1961). The results are given as means ± S.E.M. of at least ten experiments. Contamination was estimated from the amount of glucose 6-phosphatase in the fractions.

Fraction	Glucose 6-phosphatase (μg of P_i/20min per mg of protein)	Contamination of mitochondria by microsomes (%)
Microsomes	76.8 ± 2.5	—
Heavy mitochondria	5.3 ± 0.4	6.9
Light mitochondria	24.4 ± 2.67	31.8
Fluffy mitochondria	9.1 ± 0.73	11.8

Table 2. *Succinoxidase, NADH–cytochrome c reductase, malate dehydrogenase and D-β-hydroxybutyrate dehydrogenase activities of mitochondrial fractions*

The enzyme activites were measured by the procedures cited in the Materials and Methods section. The results are given as means ± S.E.M. of at least four experiments.

Mitochondrial fraction	Succinoxidase: Sp. activity (μl of O_2/20min per mg of protein)			NADH–cytochrome c reductase Sp. activity (ΔE_{550}/min per mg of protein)	Malate dehydrogenase Sp. activity (ΔE_{340}/min per mg of protein)	D-β-hydroxybutyrate dehydrogenase: Sp. activity (ΔE_{340}/min per mg of protein)		
	Cytochrome c absent	Cytochrome c present	Stimulation (%)			Unsonicated	Sonicated	Sonicated / Unsonicated ratio
Heavy	25.5 ± 2.31	80.1 ± 7.25	214	4.38 ± 0.19	1.40 ± 0.16	0.116 ± 0.013	1.015 ± 0.11	8.75
Light	17.8 ± 1.69	75.5 ± 7.81	324	4.32 ± 0.52	1.37 ± 0.17	0.262 ± 0.026	0.644 ± 0.05	2.46
Fluffy	30.0 ± 2.85	115.2 ± 10.67	284	2.29 ± 0.27	1.90 ± 0.16	0.329 ± 0.028	1.00 ± 0.11	2.59

extent of stimulation being highest for light mitochondria and higher for the fluffy particles. The specific activities of NADH–cytochrome *c* reductase in the heavy and the light mitochondrial fraction were almost identical, whereas that of the fluffy particles was less by about 50% (Table 2). For malate dehydrogenase the specific activities of heavy and light particles were almost the same and that of the fluffy particles was somewhat higher (Table 2).

D-β-Hydroxybutyrate dehydrogenase is a membrane-bound enzyme and its activity can be fully evoked only after sonication (Lehninger *et al.* 1960). Table 2 shows that unsonicated heavy mitochondria had minimum activity, whereas the light and fluffy fractions had higher activity before sonication. On sonication, the specific activities of heavy and fluffy particles were almost the same, whereas that of the light particles was less.

ADP/O *ratio of mitochondrial fractions.* Table 3 shows the results on ADP/O ratio and respiratory control index with the heavy and fluffy particles when different substrates are used. The values of ADP/O ratio are near the theoretical values for the heavy mitochondria. This mitochondrial fraction also shows a good respiratory control. On the other hand, the fluffy particles show a somewhat lower ADP/O ratio and lower respiratory control index as compared with the heavy fraction, especially when β-hydroxybutyrate is the substrate. As Table 3 shows, the decrease in respiratory control index is mainly due to increased rate of oxidation in the absence of ADP. With light particles no ADP/O ratio could be obtained with any of the substrates used, and addition of ADP resulted in a continuous increased rate of oxidation.

Mg^{2+}*-activated and 2,4-dinitrophenol-stimulated ATPase of mitochondrial fractions.* The 2,4-dinitrophenol-stimulated/Mg^{2+}-activated ATPase ratio was taken as an index of the structural integrity of mitochondria (Kielley & Kielley, 1951). Table 4 shows that heavy mitochondria have very low ATPase activity in the absence of Mg^{2+}, whereas in the presence of 2,4-dinitrophenol alone the activity increases about 26-fold. When both Mg^{2+} and 2,4-dinitrophenol are present the activity is intermediate between the Mg^{2+}-activated and the 2,4-dinitrophenol-stimulated ATPase activities. The fluffy particles have high initial ATPase activity that is practically not stimulated by Mg^{2+} but is stimulated about 2.4-fold by 2,4-dinitrophenol and is intermediate between the Mg^{2+}-activated and the 2,4-dinitrophenol-stimulated activities when both Mg^{2+} and 2,4-dinitrophenol are present. The light mitochondria have a high initial activity that remains practically unaltered under all conditions except for the slight stimulation observed in the presence of 2,4-dinitrophenol alone. Similarly

Table 3. ADP/O *ratio and respiratory control in mitochondrial fractions*

The ADP/O ratio and respiratory control index (RCI) were determined as described by Chance & Williams (1955, 1956). Q_{O_2} is given as μmol of O_2/min per mg of protein. As described in the text, no ADP/O ratio could be obtained for the light mitochondrial fraction.

	Heavy fraction				Fluffy fraction			
		Q_{O_2}				Q_{O_2}		
Substrate	ADP/O ratio	ADP present	ADP absent	RCI	ADP/O ratio	ADP present	ADP absent	RCI
Succinate	1.65	0.0341	0.0126	2.71	1.54	0.0291	0.0125	23.7
Glutamate	2.62	0.0167	0.00148	7.02	2.34	0.0195	0.0032	6.09
β-Hydroxybutyrate	2.55	0.0238	0.00210	7.05	2.38	0.0154	0.00404	3.81

Table 4. *ATPase activity of mitochondrial fractions*

The ATPase activity was determined as described by Veldsema-Currie & Slater (1968). The results are given as the means ± S.E.M. of at least four experiments.

		Sp. activity (μmol of P_i liberated/h per mg of protein)				2,4-Dinitrophenol
	$MgCl_2$	−	+	−	+	Mg^{2+}
Fraction	2,4-Dinitrophenol	−	−	+	+	ratio
Heavy		0.400 ± 0.031	1.44 ± 0.13	10.25 ± 0.51	3.63 ± 0.33	7.12
Light		2.86 ± 0.19	2.54 ± 0.19	3.66 ± 0.32	2.47 ± 0.24	1.53
Fluffy		2.71 ± 0.23	2.40 ± 0.22	6.58 ± 0.88	3.20 ± 0.31	2.79
Microsomes		2.34 ± 0.22	2.90 ± 0.07	2.64 ± 0.22	3.35 ± 0.29	0.91

the ATPase activity of microsomes is almost unaffected under all experimental conditions studied. The 2,4-dinitrophenol-stimulated/Mg^{2+}-activated ATPase ratio is highest for heavy mitochondria (7.10), is lower for fluffy particles (2.79) and is lowest for light particles (1.53).

Electrophoretic mobility of mitochondrial fractions. The results on mobility of mitochondrial fractions and their isoelectric pH values are summarized in Table 5. All mitochondrial fractions have almost the same mobilities in sucrose. Similarly the plots of pH versus mobility (Fig. 1) have in general a sigmoid form and all the mitochondrial fractions have isoelectric points in the region of pH 4.7.

Turnover of mitochondrial proteins. Fig. 2 shows that the patterns of the turnover of label are almost parallel for heavy and fluffy particles whereas the label in the light mitochondrial fraction turns over at a comparatively faster rate.

The half-lives of the heavy, light and fluffy particles have been calculated, assuming that the soluble as well as insoluble components of these mitochondrial fractions turn over at a similar rate and that the turnover rates follow first-order kinetics (Fletcher & Sanadi, 1961; Beattie *et al.* 1967). These are summarized in Table 6. The half-life values are almost the same for heavy and fluffy particles, namely 5.88 and 6.21 days respectively, whereas the light fraction turns over rapidly and has a mean half-life of 2.15 days only.

Behaviour of the mitochondrial fractions in tri-iodothyronine-treated rats

Succinoxidase activity of mitochondrial fractions. Fig. 3 shows that the succinoxidase activity of the heavy mitochondrial fraction increases maximally only after 45h after tri-iodothyronine treatment, the extent of stimulation being about 35%, in agreement with the reports of Roodyn *et al.* (1965) and Tata *et al.* (1963).

The fluffy particles show an early increase of about 35% in succinoxidase activity at 10h. The specific activity profile then forms a plateau.

The succinoxidase profile is different in the light mitochondrial fraction. The specific activity decreases considerably on thyroidectomy, and increases by about 40% or so as early as 3h after tri-iodothyronine administration. The specific activity increases still further with time after tri-iodothyronine treatment and at 10h about 100% stimulation in the activity is observed. The

Table 5. *Electrophoretic mobility and isoelectric* pH *values of mitochondrial fractions*

The experimental conditions are as described in the Materials and Methods section. The values of mobility are means ± S.E.M. of at least 20 observations. Isoelectric pH was determined from the plots of mobility versus pH (Fig. 1).

Mitochondrial fraction	Mobility (μm per V/cm per s)	Isoelectric pH
Heavy	-2.20 ± 0.092	4.75
Light	-2.20 ± 0.063	4.75
Fluffy	-2.25 ± 0.18	4.65

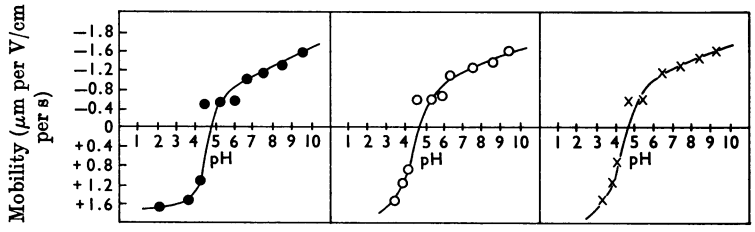

Fig. 1. Plots of mobility versus pH drawn as described by Boulter *et al.* (1965). The isoelectric pH values of mitochondrial fractions were determined from these plots. ●, Heavy fraction; ○, light fraction; × fluffy fraction.

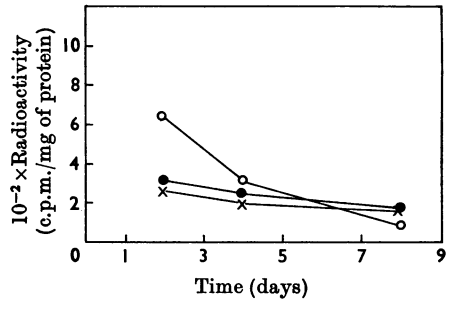

Fig. 2. Radioactivity in the mitochondrial proteins determined by the method described in the Materials and Methods section. The specific radioactivity is plotted against time to represent the turnover curves of: ●, heavy fraction; ○, light fraction; ×, fluffy fraction.

activity remains as a plateau up to 40h, decreases to some extent at 65h and remains steady at this value up to 90h.

Stimulation of homogenate succinoxidase by cytochrome c. The stimulatory effect of cytochrome *c* on the succinoxidase system (Potter, 1959) was studied with liver homogenate in order to avoid the losses of indigenous cytochrome *c* (Jacobs & Sanadi, 1960). The results are summarized in Table 7. Addition of cytochrome *c* results in 27 and 28% stimulation in unoperated and tri-iodothyronine-treated animals respectively. However, maximum stimulation (72%) is discernible in the liver homogenate from thyroidectomized rats, this being known to be deficient in respiratory units (Tata *et al.* 1963). These observations are significant since a single component of the respiratory chain could influence the succinoxidase activity of mitochondria and would mean that cytochrome *c* plays a rate-limiting role.

Malate dehydrogenase activity of the mitochondrial fractions. The activity pattern of malate dehydrogenase (Fig. 4) differs from that of succinoxidase. No stimulatory effect is observed in the specific activity of any of the mitochondrial fractions up to 40h or so after tri-iodothyronine administration. However, at the end of this period the activity in all the fractions increased simultaneously and continued to do so thereafter up to 90h, when it reached a maximum in all the fractions (5–6-fold increase).

Incorporation of DL-[1-^{14}C]*leucine into mitochondrial fractions.* The results of the incorporation of DL-[1-^{14}C]leucine into the mitochondrial fractions are summarized in Table 8. Since stimulation of protein synthesis starts at about 30h, reaches maximum at 45h and decreases thereafter by 70h after treatment with tri-iodothyronine (Roodyn *et al.* 1965), the incorporation of injected DL-[1-^{14}C]leucine was studied at these periods after tri-iodothyronine administration. The incorporation in the heavy and fluffy fractions reaches a maximum at 45h and decreases thereafter. The extent of incorporation at 45h is somewhat higher in the fluffy particles. With the light mitochondrial

Table 6. *Turnover of mitochondrial proteins*

The experimental conditions are described in the text. The values of k and $T_{\frac{1}{2}}$ were calculated as described in the Materials and Methods section, assuming that the turnover rates follow first-order kinetics.

Mitochondrial fraction	First-order reaction constant k	Standard error of k	$T_{\frac{1}{2}}$ (days)
Heavy	0.1177	0.0110	5.88
Light	0.3253	0.0104	2.15
Fluffy	0.1117	0.0267	6.21

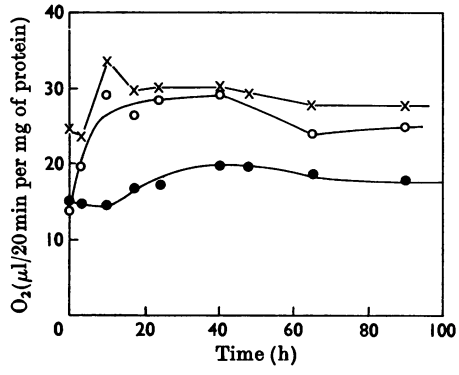

Fig. 3. Time-study of the succinoxidase activity of mitochondrial fractions as a response to single injection of tri-iodothyronine. Succinoxidase activity was measured manometrically (Potter, 1959). Other details were as given in the Materials and Methods section. ●, Heavy fraction; ○, light fraction; ×, fluffy fraction.

fraction there is very high incorporation at 30h that later shows a steady decrease with time.

Turnover of label in mitochondrial proteins. Fig. 5 shows that all mitochondrial fractions exhibit maximum incorporation on the second day, the extent of incorporation being highest in the light fraction as compared to the heavy and the fluffy fractions, which show minimum and intermediate incorporation respectively. The specific activity decreases on the fourth day by about 50% and again increases on the sixth day, especially so with the light and fluffy fractions. Thereafter a decline in the specific activity is observed up to the tenth day, the rate being more rapid for the light mitochondrial fraction.

Determination of mitochondrial proteins. Results of the quantitative determination of proteins in the mitochondrial fractions are presented in Table 9. The protein content of homogenate, total mitochondrial and heavy+fluffy fractions decreases on thyroidectomy and is restored to normal on tri-iodothyronine treatment. The changes in the specific contents of these proteins are, however, not significant. On the other hand, proteins of the light mitochondrial fraction decrease by 40% on thyroidectomy and increase by 160% on tri-iodothyronine treatment.

DISCUSSION

Heterogeneity of rat liver mitochondria. In the present studies tissue homogenization was carried out under mild and standardized conditions, with constant and reproducible results. Therefore under the conditions employed there was minimum fragmentation of mitochondria. Isolation of mitochondria in 0.25 M-sucrose containing tris and EDTA did not alter the quantitative yield of mitochondrial fractions (S. S. Katyare, P. Fatterpaker, & A. Sreenivasan, unpublished work). The fractions obtained by differential centrifugation thus appear to be discrete entities and not artifacts of preparation. Density-gradient analysis was not attempted for further purification, since there appears to be very little difference between the densities of heavy and light fractions (Gear, 1965*a*,*b*). However, it is recognized that further work, including electron-microscopic examination, is necessary for more precise characterization of these fractions.

The results of the present studies suggest that the heavy mitochondrial fraction represents well-formed structures. These particles possess all typical mitochondrial enzymes and appear to be structurally intact as judged by the D-β-hydroxybutyrate dehydrogenase activity, study of ADP/O ratio, respiratory control index and ATPase activated and stimulated by Mg^{2+} and 2,4-dinitrophenol respectively.

The fluffy particles judged by the above parameters appear to be structurally less intact. Besides, these particles also possess a high succinoxidase activity, probably due to the high succinate dehydrogenase activity associated with this fraction (Kuff & Schneider, 1954), the latter being a rate-limiting factor in the succinoxidase system (Thorn, 1962). It has been suggested that the fluffy layer may be a mixture of mitochondria and microsomes (Siekevitz, 1952; Novikoff, Podber, Ryan & Noe, 1953), a view also favoured by Getz, Bartley, Stirpe, Notton & Renshaw (1962) on the basis of the lipid composition of this fraction. Our results on

Table 7. *Effect of cytochrome* c *on succinoxidase activity of liver homogenate*

The animals received a single injection of tri-iodothyronine (20 μg/100 g body wt.) as described in the Materials and Methods section. The succinoxidase activity was determined manometrically as described in Table 2, except that the amount of homogenate protein was about 9.5–10.5 mg (0.5 ml of 10% liver homogenate in 0.25 M-sucrose). The results are given as means ± S.E.M. of four independent experiments.

State of animals	Sp. activity (μl of O_2/20 min per mg of protein)		Stimulation (%)
	Cytochrome *c* present	Cytochrome *c* absent	
Control	15.78 ± 0.18	20.05 ± 0.17	27
Thyroidectomized	14.02 ± 0.75	23.61 ± 1.60	72
+ Tri-iodothyronine (48h)	16.95 ± 0.42	21.69 ± 0.38	28

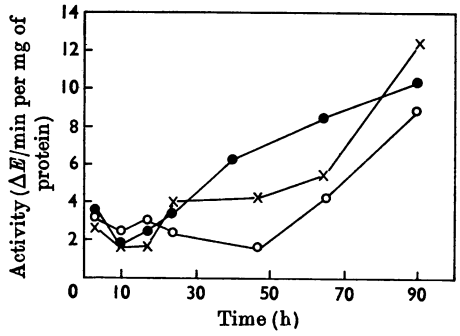

Fig. 4. Malate dehydrogenase activity of mitochondrial fractions as a time-response to tri-iodothyronine treatment. The enzyme activity was determined by the method of Ochoa (1955). Other details are as given in the Materials and Methods section. ●, Heavy fraction; ○, light fraction ×, fluffy fraction.

the enzyme profiles of this fraction, obtained under controlled homogenization and isolation procedure and after correction for microsomal contamination, indicate that this mitochondrial fraction parallels the heavy mitochondria in almost all respects and probably represents the population containing damaged mitochondria.

The light mitochondrial fraction is contaminated with microsomes to the extent of about 30%. However, correction for this in terms of the marker enzyme, glucose 6-phosphatase, may be reasonably expected not to influence its distinctive properties. Such corrections have been applied by several workers (Beattie, 1968*b*; Volfin, Kaplay & Sanadi, 1969).

The light mitochondrial fraction also possesses all the characteristic mitochondrial enzymes, but is unable to carry out coupled oxidative phosphorylation and possesses a 2,4-dinitrophenol-insensitive ATPase, similar to that of the microsomes (Table 4). In view of the role of ATPase as a coupling factor (Pullman, Penefsky, Datta & Racker, 1960), the inability of these particles to carry out coupled oxidative phosphorylation may be due to non-participation of ATPase as a coupling factor or lack of other coupling factor(s). However, this possibility has not been fully examined in the present studies.

The study of mobility and isoelectric pH indicates that the basic membrane structure contributing to the charge on mitochondria may be similar to all the fractions studied.

The study of turnover rates indicates that the heavy, fluffy and light particles have half-lives of 5.88, 6.21 and 2.15 days respectively. The first two fractions have almost the same half-lives, although this value is somewhat lower than the one reported by earlier workers (Fletcher & Sanadi, 1961; Beattie *et al.* 1967). Since the turnover studies in the present investigation were carried out for a comparatively shorter period up to 10 days after injection of DL-[1-^{14}C]leucine, the observed low values may be due to a time-effect. However, the fact remains that the light mitochondrial fraction has a comparatively very fast turnover and shorter half-life, that is also distinctly different from and not in between those of heavy mitochondria (5.88 days) and microsomes (4.8 days; S. S. Katyare, P. Fatterpaker & A. Sreenivasan, unpublished work).

Effect of tri-iodothyronine administration on the behaviour of mitochondrial fractions. The differential behaviour of the three mitochondrial fractions has been further magnified by the treatment of thyroidectomized animals with tri-iodothyronine, a condition favouring mitochondrial protein synthesis. As is evident from the results in Tables 8 and 9 and Figs. 3 and 5, the three mitochondrial fractions behave quite differently as a response to tri-iodothyronine. Of note is the fact that it is the light fraction that is affected maximally on thyroidectomy and subsequent treatment with tri-iodothyronine. Thus succinoxidase activity in this fraction is maximally affected by thyroidectomy

Table 8. DL-[1-^{14}C]*Leucine incorporation into mitochondrial fractions*

The animals received a single injection of tri-iodothyronine (20 μg/100 g body wt.) as described in the Materials and Methods section. DL-[1-^{14}C]Leucine was injected intraperitoneally at 30, 45 and 70 h after tri-iodothyronine administration and the animals were killed 2 h thereafter. The radioactivity in the mitochondrial fractions was counted in a liquid-scintillation spectrometer. Other details are described in the text. The results are typical of three sets of experiments.

	[^{14}C]Leucine incorporation (c.p.m/mg. of protein)				
			+Tri-iodothyronine		
Mitochondrial fraction	Control	Thyroidectomized	30 h	45 h	70 h
Heavy	317	185	561	883	691
Light	619	493	1113	668	481
Fluffy	314	313	632	1346	839

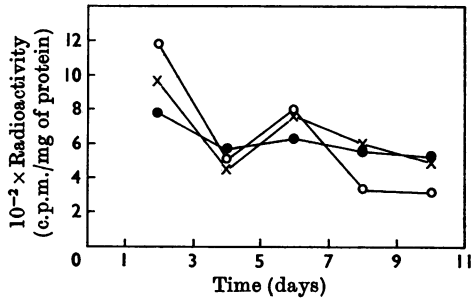

Fig. 5. Turnover of [1-^{14}C]leucine in the proteins of the mitochondrial fractions from animals receiving sustained tri-iodothyronine treatment. Other details are as given in the Materials and Methods section. ●, Heavy fraction; ○, light fraction; ×, fluffy fraction.

(see Table 2 and Fig. 3 for comparison) and is stimulated as early as 3 h after tri-iodothyronine treatment. In view of the stimulatory effect of cytochrome *c* on the succinoxidase system (Table 7), the early stimulation of succinoxidase activity of this fraction would mean that the essential respiratory units may be added much earlier to this fraction as compared with the other two fractions. Such transfer of cytochrome *c* to mitochondria from an extramitochondrial site of synthesis has been demonstrated by several workers (González-Cadavid & Campbell, 1967; Kadenbach, 1967*b*; Freeman, Haldar & Work, 1967). These results are also consistent with the observations by Roodyn *et al.* (1965) on a selective synthesis of respiratory units by treatment with thyroid hormone. On the other hand, the observations on malate dehydrogenase activity (Fig. 4) would mean that the soluble tricarboxylic acid-cycle enzymes may be synthesized at a later stage. The possibility of a sequential integration of soluble proteins has been suggested by Beattie (1968*a*) from studies *in vitro* on leucine incorporation into the mitochondrial proteins of rat liver slices. She has observed that the specific radioactivity of water-soluble proteins containing, among other things, malate dehydrogenase and glutamate dehydrogenase increased by the largest percentage during the first 30 min after the 'chase' addition, and there was a linear increase in the specific radioactivity of the potassium chloride-soluble fraction containing cytochrome *c* for 90 min after the 'chase' addition. Our results, however, are contrary to these observations in that the stimulation of succinoxidase activity precedes that of malate dehydrogenase. This may perhaps be ascribed to differences in experimental conditions and also to the influence of thyroid hormone.

In view of the increased ability of the light fraction to oxidize substrates on treatment with tri-iodothyronine (Fig. 3), its ability to carry out oxidative phosphorylation with succinate as the substrate was tested polarographically as well as manometrically in separate experiments. These experiments have indicated that, even under the condition that enhances the rate of oxidation, the capacity for oxidative phosphorylation is not conferred on these particles (S. S. Katyare, P. Fatterpaker & A. Sreenivasan, unpublished work).

The pattern of incorporation of labelled leucine into the mitochondrial fractions (Table 8) indicates similarity in the behaviour of the heavy and fluffy fractions. On the other hand, the light mitochondria fraction shows considerably higher incorporation at 30 h, when the extent of incorporation in the other two fractions is much less. These results therefore suggest an initial rapid rate of synthesis and higher synthetic ability in this fraction.

The turnover profiles of mitochondrial proteins in animals receiving repeated doses of tri-iodothyronine (Fig. 5) are remarkably different from those observed with the mitochondrial fractions from normal rat liver (Fig. 2), and do not follow first-order kinetics. The specific radioactivity of the

Table 9. *Proteins of the subcellular fractions of liver*

The animals received a single injection of tri-iodothyronine (20 μg/100 g body wt.) and were killed after 48 h. Mitochondrial fractions were isolated quantitatively as described in the Materials and Methods section. The protein contents are expressed as percentages of the control, the values expressed as mg/g liver (wet wt.) being: homogenates, 200.0±10.0; total mitochondria, 41.8±3.6; heavy and fluffy fraction, 40.6±2.7; light mitochondria, 0.8±0.04. The results are given as means±S.E.M. of six experiments.

	Protein content (% of control values)			
State of animals	Homogenate	Total mitochondria	Heavy and fluffy fraction	Light mitochondria
Thyroidectomized	87.5±6.23	94.8±2.81	95.4±2.32	62.5± 6.20
+Tri-iodothyronine (48 h)	107.5±6.12	105.0±2.10	102.0±3.86	264.0±10.21

label, instead of decreasing with time, increases on the sixth day, especially in the light fraction and to a smaller extent in the fluffy fraction (Fig. 5). This increased specific radioactivity could arise either through increased protein synthesis or by accretion of preformed labelled proteins. The first possibility, however, seems unlikely in view of the fact that most of the [1-^{14}C]leucine is incorporated within 2–3 h after injection. Although label could also arise out of degradation of labelled subcellular proteins, the amounts of light and fluffy mitochondria being a fraction of the total cellular proteins, the possibility of reutilization of the label by these particles would be comparatively less. Incorporation of preformed protein units therefore seems most likely. Transfer of soluble proteins from an extramitochondrial site as demonstrated by several workers (González-Cadavid & Campbell, 1967; Kadenbach, 1967*a*,*b*; Freeman *et al.* 1967; Beattie, Basford & Koritz, 1966) favours such an assumption.

The decrease in the content of light-mitochondrial proteins on thyroidectomy and an increase on the second day after tri-iodothyronine treatment is significant and is consistent with the high synthetic ability (Table 8), rapid turnover (Figs. 2 and 5) and short half-life of about 2.15 days (Table 6) of these mitochondria.

Mitochondrial heterogeneity in relation to mitochondrial genesis. It is now generally accepted that the mitochondrial proteins are synthesized under the dual control of mitochondrial DNA and nuclear DNA (Work, Coote & Ashwell, 1968; Roodyn & Wilkie, 1968). Beattie (1968*a*) has suggested that biosynthesis of mitochondria *in vivo* appears to require initial synthesis of the outer membrane and the insoluble proteins of the inner membrane before the incorporation of soluble matrix proteins. In the present studies we have shown the transfer of preformed soluble protein units to the light-mitochondrial fraction, as well as a rapid turnover and high synthetic ability of this fraction. Whether the light-mitochondrial fraction represents the precursors for mitochondrial genesis *in vivo* as suggested by Beattie (1968*a*), or whether they represent the counterparts of the promitochondria of anaerobically grown yeast (Criddle & Schatz, 1969; Paltauf & Schatz, 1969; Plattner & Schatz, 1969), will be borne out only by more direct experiments. However, the present results suggest that the light-mitochondrial fraction probably represents the premature structures which by accretion of essential proteins mature into the stable heavy mitochondria. The fluffy fraction, however, shows behaviour intermediate between that of heavy and light mitochondrial fractions and appears to be a mixture of regenerating and degenerating mitochondria with microsomes.

REFERENCES

Bangham, A. D., Flemans, R., Heard, D. H. & Seaman, G. V. F. (1958). *Nature, Lond.*, **182**, 642.

Beattie, D. S. (1968*a*). *J. biol. Chem.* **243**, 4027.

Beattie, D. S. (1968*b*). *Biochem. biophys. Res. Commun.* **31**, 901.

Beattie, D. S., Basford, R. E. & Koritz, S. B. (1966). *Biochemistry, Easton*, **5**, 926.

Beattie, D. S., Basford, R. E. & Koritz, S. B. (1967). *J. biol. Chem.* **242**, 4584.

Beaufay, H., Jaques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. & de Duve, C. (1964). *Biochem. J.* **92**, 184.

Boulter, D., Douglas, H. W. & Laycock, M. V. (1965). *New Physiol.* **64**, 443.

Chance, B. & Williams, G. R. (1955). *Nature, Lond.*, **175**, 1120.

Chance, B. & Williams, G. R. (1956). *Adv. Enzymol.* **17**, 65.

Corwin, L. W. & Schwarz, R. (1959). *J. biol. Chem.* **234**, 191.

Criddle, R. S. & Schatz, G. (1969). *Biochemistry, Easton*, **18**, 322.

de Duve, C. (1967). In *Methods in Enzymology*, vol. 10, p. 7. Ed. by Eastabrook, R. W. & Pullman, M. E. New York: Academic Press Inc.

Fatterpaker, P., Bhuvaneswaran, C., Patwardhan, M. V. & Sreenivasan, A. (1965). In *Proc. I.C.R.O. int. Conf. Cell Biology, Bombay*, p. 445. Bombay University Press.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Fletcher, M. J. & Sanadi, D. R. (1961). *Biochim. biophys. Acta*, **51**, 356.

Freeman, K. B., Haldar, D. & Work, T. S. (1967). *Biochem. J.* **105**, 947.

Freeman, K. B., Roodyn, D. B. & Tata, J. R. (1963). *Biochim. biophys. Acta*, **72**, 129.

Frisell, W. R., Patwardhan, M. V. & Mackenzie, C. G. (1965). *J. biol. Chem.* **240**, 1829.

Gear, A. R. L. (1965*a*). *Biochem. J.* **95**, 118.

Gear, A. R. L. (1965*b*). *Biochem. J.* **97**, 532.

Getz, G. S., Bartley, W., Stirpe, E., Notton, B. & Renshaw, A. (1962). *Biochem. J.* **83**, 181.

González-Cadavid, N. F. & Campbell, P. N. (1967). *Biochem. J.* **105**, 443.

Gustafsson, R., Tata, J. R., Lindberg, O. & Ernster, L. (1965). *J. Cell Biol.* **26**, 555.

Jacobs, E. E. & Sanadi, D. R. (1960). *J. biol. Chem.* **235**, 531.

Kadenbach, B. (1967*a*). *Biochim. biophys. Acta*, **134**, 430.

Kadenbach, B. (1967*b*). *Biochim. biophys. Acta*, **138**, 651.

Kielley, W. W. & Kielley, R. K. (1951). *J. biol. Chem.* **191**, 485.

Kuff, E. I. & Schneider, W. C. (1954). *J. biol. Chem.* **206**, 677.

Lehninger, A. L., Sudduth, H. C. & Wise, J. B. (1960). *J. biol. Chem.* **235**, 2450.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Maclagan, N. F. & Sheahan, M. M. (1950). *J. Endocr.* **6**, 456.

Mans, R. J. & Novelli, G. D. (1961). *Archs Biochem. Biophys.* **94**, 48.

Matile, Ph. & Bahr, G. F. (1968). *Expl Cell Res.* **52**, 301.

Michaelis, L. (1931). *Biochem. Z.* **234**, 139.

Novikoff, A. B., Podber, E., Ryan, J. & Noe, L. (1953). *J. Histochem. Cytochem.* **1**, 27.

Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 735. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Paltauf, F. & Schatz, G. (1969). *Biochemistry, Easton*, **8**, 335.

Plattner, H. & Schatz, G. (1969). *Biochemistry, Easton*, **8**, 339.

Potter, V. R. (1959). In *Manometric Techniques*, 3rd ed., p. 174. Ed. by Umbreit, W. W., Burris, R. H & Stauffer, J. F. Minneapolis: Burgess Publishing Co.

Pullman, M. E., Penefsky, H. S., Datta, A. & Racker, E. (1960). *J. biol. Chem.* **235**, 3330.

Recknagel, R. O. & Lombardi, B. (1961). *J. biol. Chem.* **236**, 564.

Roodyn, D. B., Freeman, K. B. & Tata, J. R. (1965). *Biochem. J.* **94**, 628.

Roodyn, D. B. & Wilkie, D. (1968). *The Biogenesis of Mitochondria*, pp. 51, 52. London: Methuen and Co. Ltd.

Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.

Tata, J. R., Ernster, L., Lindberg, O., Arrhenius, E., Pederson, S. & Heldman, R. (1963). *Biochem. J.* **86**, 408.

Thorn, M. B. (1962). *Biochem. J.* **85**, 116.

Veldsema-Currie R. D. & Slater, E. C. (1968). *Biochim. biophys. Acta*, **162**, 310.

Volfin, P., Kaplay, S. S. & Sanadi, D. R. (1969). *J. biol. Chem.* **244**, 5631.

Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.

Work, T. S., Coote, J. L. & Ashwell, M. (1968). *Fedn Proc. Fedn Am. Socs exp. Biol.* **27**, 1174.