Some Features of Mitochondria and Fluffy Layer in Regenerating Rat Liver

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1. Mitochondria and fluffy layer were prepared from control and regenerating rat liver. Differential and density-gradient centrifugation were used to fractionate the preparations, which were examined for protein content, density and the activity of cytochrome *c* oxidase, succinate dehydrogenase, NAD-isocitrate dehydrogenase and NADP-isocitrate dehydrogenase. 2. During regeneration the mitochondrial protein content of the liver fell by 18% from the control value of 18.4 mg. of protein/g. of liver (wet wt.) and by 3 weeks had risen to 130% of the control value. It then declined slowly. 3. The fluffy-layer protein content (4.7 mg./g. of liver) varied inversely as the mitochondrial content and increased by 70% in the early stages (10 days) of liver regeneration. The results suggest that fluffy layer may partially represent both partly formed and broken-down mitochondria. 4. NAD- and NADP-isocitrate dehydrogenases differed in their behaviour during liver regeneration. 5. The succinate-dehydrogenase and NADP-isocitrate-dehydrogenase activity of fluffy layer was high and rose during the early stages of liver regeneration (1 week). Succinate dehydrogenase and cytochrome *c* oxidase were concentrated in the lighter fluffy-layer particles 10 days to 3 weeks after partial hepatectomy. The significance of this with respect to mitochondrial formation is discussed. 6. Mitochondrial fractions possessed a certain degree of heterogeneity in enzymic activity when separated according to size and density. The mean density of heavy mitochondria was 1.198, light mitochondria 1.193. Fluffy layer was nearly homogeneous in control liver, but during regeneration considerable heterogeneity became evident. The significance of the heterogeneity is discussed.

Mitochondria have a fairly short life. This has been estimated as a half-life of 10.3 days (Fletcher & Sanadi, 1961) by measuring the rate of loss of radioactivity of mitochondria labelled with [³⁵S]methionine and [¹⁴C]acetate. The mitochondria appeared to be broken down as a unit. There is considerable speculation about the mechanism of mitochondrial genesis and several theories have been set forward: (a) 'de novo' origin (Beckwith, 1914; Harvey, 1953; Dempsey, 1956); (b) nuclear membrane origin (Hartman, 1954); (c) microbody origin (Gansler & Rouiller, 1956; Rouiller & Bernard, 1956); (d) division (Lund, Vater & Hanson, 1958; Luck, 1963).

Bearing in mind a turnover of mitochondria, it was thought there should exist a spread of mitochondria in terms of age, and that this spread would be reflected in variation of chemical make-up and enzymic activity. In other words there may be a population of both different age and type. Such an idea is not new (Fletcher & Sanadi, 1961), but has

not as yet been fully substantiated. Kuff & Schneider (1954) illustrated some degree of mitochondrial heterogeneity with respect to one enzyme, succinate dehydrogenase.

The extent of mitochondrial turnover is about 5% per day, and to elucidate some of the features of mitochondrial genesis would entail determining what special characteristics, if any, there are in the small section of the total population representing rapidly growing mitochondria. To increase the chances of observing these characteristics, regenerating rat liver was chosen as a convenient tissue. At 2-3 weeks after partial hepatectomy the liver has doubled its weight (Harkness, 1957) concomitantly with a large synthesis of mitochondria. The processes concerned with mitochondrial formation must consequently be considerably activated in comparison with resting liver, and it was decided to investigate a physical, a chemical and some enzymic characteristics of mitochondria during the course of liver regeneration. These might indicate how the mitochondrial structure is elaborated.

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Use is made of a combination of differential and density-gradient centrifugation for studying protein content, and of the activities of four enzymes: cytochrome *c* oxidase, succinate dehydrogenase, NAD- and NADP-isocitrate dehydrogenases.

EXPERIMENTAL

Chemicals. Cytochrome *c*, type III, was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; ascorbic acid from Roche Products Ltd., Welwyn Garden City, Herts.; nicotinamide nucleotides and sodium succinate from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; glucose 6-phosphate (barium salt), INT* and semicarbazide hydrochloride from British Drug Houses Ltd., Poole, Dorset; potassium dihydrogen L_+ -isocitrate was a gift from Dr H. B. Vickery. All other reagents were of A.R. grade, or of the highest purity commercially available. Phosphate buffers were made from the potassium salts.

Animals. Male albino rats of the Wistar strain were used throughout. The animals weighed from 200 to 300 g.

Partial hepatectomy. This was carried out as described by Higgins & Anderson (1931). Rats were anaesthetized with ether, and given 10% (w/v) glucose solution for a day after operation. They were then kept on a normal diet until killed.

Mitochondrial preparation. Rats were killed by stunning and by dislocating the neck. The livers were removed as quickly as possible and cooled in crushed ice for 5 min. They were then passed through a Fisher mincer, the resulting pulp was weighed and the mitochondria and fluffy layer were isolated in 0.25 M-sucrose, pH 7.4, as described by Werkheiser & Bartley (1957). 'Light' mitochondria are defined as those sedimenting between 6000 and 10000 g-min. and 'heavy' mitochondria as those sedimenting between 3400 and 6000 g-min. The fluffy layer was obtained by a technique similar to that described by Werkheiser & Bartley (1957). After sedimenting the impure mitochondria from the supernatant, the mitochondrial residues were washed twice by resuspension in 1.5 vol. of sucrose and centrifuging for 10 min. at 10000 g. After each washing, the supernatants were discarded and the pinkish, freely flowing residues of fluffy layer were collected. It was difficult to obtain a fluffy layer free from mitochondrial contamination, and constancy of technique in pouring off the layer was attempted throughout. When the lighter, buff-coloured mitochondria just began to reach the tip of the centrifuge tube, pouring was stopped. The much smaller amount of fluffy layer separating on the second mitochondrial washing was poured off in a similar manner. The fractions were finally resuspended in 1.5 vol. of sucrose and centrifuged at 17000 g. The mitochondrial and fluffy-layer pellets were finally suspended in 0.25 M-sucrose before density-gradient centrifugation.

Density-gradient centrifugation. This was carried out in the SW 25 head of the Spinco L preparative ultracentrifuge. Gradients were obtained by layering 3 ml. samples of aq. sucrose solutions from a density of 1.230 (1.8 M-sucrose) to 1.155 (1.2 M-sucrose). In all, seven layers were set up, and a bottom layer of 2.5 M-sucrose covered the round base of the

Spinco tube. The gradients were allowed to stabilize for at least 3 hr. before centrifugation. A portion (2 ml.) of the mitochondrial suspension was layered immediately before centrifuging, this being for 90 min. at 25000 rev./min. (57.6×10^5 g-min.). The various fractions were removed, 3 ml. at a time, by means of controlled, gentle sucking with a polythene tube attached to a Pro-pipette. The tube's tip was bent to direct the sucking force sideways; this system allows for minimum disturbance of lower layers and gave good reproducibility of results. The various fractions were adjusted to 0.25 M with glass-distilled water, and the mitochondria and fluffy layer were spun down (25000 g-min.). The mitochondria and fluffy layer were finally resuspended in 0.1 M-potassium phosphate buffer, pH 7.4, and stored overnight at 0–2°. These solutions were then used for the enzyme assays on the following day. All the other operations described were carried out at 0–5°. To test the stability of the enzymes at 0–2°, assays were carried out immediately after preparation of the mitochondria, and then again the following day at the times used during the experiments. The activity remaining for cytochrome *c* oxidase was 96%; for succinate dehydrogenase, 102%; for NAD-isocitrate dehydrogenase, 90%; for NADP-isocitrate dehydrogenase, 96%. These are average values for the three fractions.

Assay of enzymic activities. (a) Cytochrome *c* oxidase (Minnaert, 1961). The partially reduced cytochrome *c* used in the assay was prepared as follows. A solution of cytochrome *c* (0.5 mM) was reduced by adding a little less than the equivalent amount of ascorbic acid. The reduction was followed spectrophotometrically by measuring the ratio of the extinction at 550 m μ and 565 m μ . This should be about 10, and addition of more ascorbic acid should produce only a slight alteration of this figure. The percentage reduction of cytochrome *c* is about 90%.

The assay of cytochrome *c* oxidase was carried out with a Zeiss PMQ 11 spectrophotometer. The change in extinction (ΔE) of reduced cytochrome *c* at 550 m μ was measured. Mitochondria were incubated at 25° in the following medium (final vol. 3.0 ml.) in glass cuvettes of 1 cm. light-path: potassium phosphate buffer (pH 6.9), 100 mM; EDTA, 1 mM; partially reduced cytochrome *c*, 16 μ M. The incubations were initiated by the addition of the mitochondrial suspension to the complete incubation medium. ΔE was followed at 30 sec. intervals for 3 min. A portion (0.1 ml.) of aq. 3% (w/v) ferricyanide solution was then added. This completely oxidized the cytochrome *c*, and the extinction of the system was then measured. A plot of $\log \Delta E$ between the fully oxidized and partially oxidized cytochrome *c*, against time, is a straight line, the slope of which is proportional to the cytochrome *c*-oxidase activity. The specific activity for the enzyme is defined as negative $\ln \Delta E$ /min./mg. of mitochondrial protein.

(b) Succinate-INT reductase. This was measured as described by Pennington (1961), except that the incubation times were usually 10 min.

(c) NAD- and NADP-isocitrate dehydrogenases. For measuring NAD-isocitrate dehydrogenase activity mitochondria were incubated at 37° in the following medium (final vol. 1.0 ml.) in 10 ml. glass-stoppered tubes: potassium phosphate buffer (pH 7.4), 50 mM; semicarbazide, 20 mM; monopotassium disodium isocitrate, 5 mM; INT, 0.1% (w/v); NAD, 1 mM; sucrose, 25 mM. Trichloroacetic acid (1 ml. of 10%, w/v) was added and the formazan was extracted with 4 ml. of ethyl acetate and its extinction measured at 490 m μ .

* Abbreviation: INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

For the NADP-isocitrate dehydrogenase assay, the concentration of NADP giving maximal activity was 0.1 mM.

The specific activity for enzymes assayed for tetrazolium reduction is defined as $\Delta E/\text{min./mg.}$ of mitochondrial protein/ml. of incubation mixture, the formazan being extracted and read in 4 ml. of ethyl acetate.

(d) Glucose 6-phosphatase (Harper, 1963). Washed mitochondria and fluffy layer were prepared as described above and, as well, an impure nuclear fraction (material sedimenting before the heavy mitochondria) and microsomal and supernatant fractions. The microsomes were obtained by centrifuging the supernatant and the mitochondrial and fluffy-layer washings at $3 \times 10^6 \text{ g. min.}$ in the 40 rotor of the Spinco L preparative ultracentrifuge. The activity was estimated within 4–5 hr. of killing the rats.

Estimation of protein. A variation of the biuret method of Ellman (1962) was used. Samples of the mitochondrial suspensions were brought to a final concentration of 8% (w/v) trichloroacetic acid. The precipitate was washed twice with water and once with ether, and left overnight in 2N-NaOH. The resultant solution was cleared by washing with hexane. Two series of tubes were set up: 0.4 ml. of 6N-NaOH in one, and 0.4 ml. of 6N-NaOH containing 0.12% (w/v) of CuSO_4 in the other. A portion (0.8 ml.) of the protein solutions containing 10–150 $\mu\text{g.}$ of protein was added to each pair, the solutions were well mixed and the extinctions at 263 m μ measured after 30 min., each series being read against the respective blank. From the difference in extinction, the protein content was obtained by reference to a protein standard curve (bovine plasma albumin).

To test whether various fractions gave the same colour yield, well-washed trichloroacetic acid precipitates of the fractions were prepared, dried and weighed. They were dissolved in 2N-NaOH and protein was estimated. The protein/dry wt. ratios were taken, the result being expressed with the heavy mitochondrial ratio as unity. The value for light mitochondria was 0.96, and for fluffy layer 0.97.

RESULTS

Increase in mitochondria during regeneration. The increase in liver wet weight, together with the increase in mitochondrial and fluffy-layer protein, are shown in Table 1. Three separate experiments were carried out, two rats (their livers being pooled) being

used at each time-interval. It was observed that after a slight lag period the liver increased rapidly in weight between 5 and 10 days. The rate of increase lessened by 15 days after operation, and the final increase in weight was within 5% of the amount removed by partial hepatectomy. This is in agreement with Harkness (1957). The increase in mitochondrial protein did not follow the same characteristics; maximum increase occurred 8–18 days after operation with a tailing off at 22 days after partial hepatectomy. The increase in mitochondrial protein was greater than the amount removed by the operation. The variations in fluffy-layer protein content showed a very different form. Between 2 and 10 days there was a large increase (from 54 to 157% of the control), between 10 and 22 days a gradual fall and, finally, a sharp rise occurred.

These values refer to the overall changes during regeneration, but perhaps more instructive are the changes in mitochondrial protein content on a weight basis, i.e. specific content. This gives an indication of the amount of mitochondria in a given weight of liver. This may be interpreted to be an indication of either mitochondrial numbers or mitochondrial mass or both. The results are given in Table 1. The mitochondrial content fell slightly for 10 days, after which there was a sharp rise to give a plateau at 3 weeks after partial hepatectomy. In this context it can be noted that 2 days after partial hepatectomy Allard, Mathieu, de Lamirande & Cantero (1952) detected a fall of some 20% in the number of mitochondria in a given weight of fresh liver, or alternatively a fall of about 15% in the number of mitochondria per cell.

At 3 weeks after partial hepatectomy the mitochondrial content was about 30% higher than normal. In contrast, the fluffy-layer specific content rose sharply to give a maximum at 10 days and then fell to near the control value at 22 days, after which an increase was recorded.

Changes in total mitochondrial enzymic activity during regeneration. These have been calculated by

Table 1. *Changes in liver weight and mitochondrial and fluffy-layer protein during liver regeneration*

Values are from three experiments, expressed as percentages of the control and as means $\pm$ S.D. The specific contents refer to the amount of mitochondria, or fluffy layer, in a given weight of liver.

Days after partial hepatectomy	Liver (wet wt.)	Total mitochondrial protein	Fluffy-layer protein	Mitochondrial specific content	Fluffy-layer specific content
Control	100	100	100	100	100
2	39 $\pm$ 2	35 $\pm$ 1	54 $\pm$ 5	96	122
6	65 $\pm$ 8	46 $\pm$ 3	98 $\pm$ 6	82	145
10	85 $\pm$ 10	76 $\pm$ 4	157 $\pm$ 8	95	172
15	89 $\pm$ 8	93 $\pm$ 1	129 $\pm$ 15	112	133
22	101 $\pm$ 4	127 $\pm$ 10	110 $\pm$ 5	132	102
31	98 $\pm$ 9	131 $\pm$ 7	201 $\pm$ 35	140	192

Table 2. *Changes in enzyme activity during liver regeneration*

Total activity is determined by summing the activities of the individual fractions separated from the sucrose-density gradient. The activity of each fraction (per g. of liver wet wt.) is found by multiplying the specific activity for the given enzyme (for definition see the Experimental section) by the total amount of mitochondrial or fluffy-layer protein present in that fraction. All values are expressed as percentages of their respective controls. Values for cytochrome *c* oxidase represent the mean for two separate experiments; those for the other enzymes refer to one experiment. (a) Combined heavy- and light-mitochondrial fractions; (b) fluffy layer.

Days after partial hepatectomy	Cytochrome <i>c</i> oxidase		Succinate dehydrogenase		NAD-isocitrate dehydrogenase		NADP-isocitrate dehydrogenase	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Control ...	100	100	100	100	100	100	100	100
2	92	108	122	165	72	48	104	156
6	99	108	100	220	45	52	83	275
10	138	108	89	120	51	62	109	142
15	142	100	82	120	100	86	83	79
22	230	105	124	80	70	29	96	53
31	165	177	91	210	84	105	78	157
42	100	77	—	—	—	—	—	—

Table 3. *Recovery of enzymic activity after density-gradient centrifugation*

Values are expressed as percentages of the total activity before density-gradient centrifugation.

Fraction	Cytochrome <i>c</i> oxidase	Succinate dehydrogenase	NAD-isocitrate dehydrogenase	NADP-isocitrate dehydrogenase
Heavy mitochondria	72	72	75	87
Light mitochondria	86	75	84	82
Fluffy layer	80	82	113	62

summing the activities of the individual fractions separated from the density gradient, and are summarized in Table 2. The values are given as activity/g. of liver (wet wt.). The activity of each fraction is determined by multiplying the specific activity for the given enzyme by the total amount of mitochondrial protein present in that fraction.

The possibility of enzymic inactivation by high sucrose concentration in the density gradient was tested. Samples of the mitochondrial and fluffy-layer suspensions before density-gradient centrifugation were kept and their activity was assayed at the same time as those of the fractions prepared after density-gradient centrifugation. The recoveries, expressed as percentages, are given in Table 3.

(a) Cytochrome *c* oxidase. The mitochondrial content remained nearly constant for 6 days, after which an increase (130%) to a maximum at 3 weeks occurred. By 6 weeks the content had returned to the control value. The fluffy-layer content remained constant for 3 weeks before an increase at 30 days and by 6 weeks it had fallen to below the control value.

(b) Succinate dehydrogenase. The content for mitochondrial succinate dehydrogenase did not alter significantly during the course of liver regeneration. In contrast, the fluffy-layer content increased by over 100% at 6 days after partial

hepatectomy; it then fell to near the control value and finally increased at 30 days. The increase was similar to that shown by cytochrome *c* oxidase.

(c) NAD- and NADP-isocitrate dehydrogenases. These two enzymes showed very different changes during regeneration. For the NAD enzyme, the general direction of changes for mitochondrial and fluffy-layer content were similar. This behaviour was not shown by any of the other enzymes studied. The total activity in both fractions fell rapidly to give a minimum at 6 days after partial hepatectomy; there was a peak at 2 weeks and, by the end of the experiment, the specific content had risen to near the control value. For the NADP enzyme, the mitochondrial content remained almost constant throughout regeneration, and the fluffy-layer content showed a striking increase of nearly 200% at 6 days after the operation and then returned to near the control value.

The observation that the succinate dehydrogenase content of mitochondria increased slightly 2 days after partial hepatectomy is contrary to that of Novikoff & Potter (1948), who demonstrated a marked decrease in the activity of this enzyme. They employed manometric measurements on whole homogenates.

Distribution of mitochondrial and fluffy-layer protein in the density gradient. The effect of regeneration

Table 4. *Distribution of mitochondrial and fluffy-layer protein along the density gradient*

Results are expressed as percentages of total protein.

	Sucrose gradient molarity	Density	Normal liver	Days after partial hepatectomy					
				2	6	10	15	22	31
Heavy and light mitochondria	1.3	1.167	0.8	1.4	1.9	1.7	0.6	0.6	0.4
	1.4	1.180	16.8	18.1	34.5	41.6	26.7	26.3	10.3
	1.5	1.192	43.7	41.7	38.0	33.6	48.4	45.0	46.5
	1.6	1.205	24.6	25.7	15.7	13.5	13.8	18.2	26.1
	1.7	1.215	9.9	9.8	6.3	6.1	7.0	6.0	11.1
	1.8	1.230	4.1	3.2	3.7	3.2	3.4	3.8	5.7
Fluffy layer	1.3	1.167	3.1	6.3	6.8	4.6	3.3	4.5	1.7
	1.4	1.180	18.4	19.3	29.4	25.2	26.0	16.6	7.9
	1.5	1.192	31.8	28.4	25.2	21.8	24.7	24.4	38.1
	1.6	1.205	22.7	23.2	21.8	19.1	18.4	20.0	26.9
	1.7	1.215	14.5	12.6	10.5	18.1	13.3	19.2	15.9
	1.8	1.230	9.6	10.2	6.2	11.1	14.3	15.2	9.5

on the density of mitochondria and fluffy layer is given in Table 4 and may also be seen in Figs. 1-12. The values for the various fractions along the density gradient are expressed as % of total protein. The distribution for both fractions is Gaussian and is similar to that described by Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959). The mean density of the combined mitochondrial fractions (1.196) agrees with that (1.192) of Beaufay (1962) and Schuel, Tipton & Anderson (1964), who used a similar technique of density-gradient centrifugation. An interesting point, however, is that the heavy and light mitochondria differ in density, the former being 1.198 and the latter 1.193. The mean density of fluffy layer (1.194) lies closer to that of light mitochondria. Points that may be noted are: particles of density 1.167-1.18 increased in amount during the early stages of regeneration, light mitochondria and fluffy layer preceding the shift of heavy mitochondria; after 10 days there was a decrease and, by the end of the experiment, the amount present in the lighter fractions had fallen to below the control value. After 2 weeks the concentration of the particles present in the denser regions, density 1.205-1.215, increased.

Changes in enzyme distribution during regeneration. These have been determined for heavy and light mitochondria and for fluffy layer, for both control and regenerating rat liver. Six fractions corresponding to sucrose densities 1.3-1.8M were taken from the density gradient and the results are reported in Figs. 1-12, the % of total enzyme activity and % of total protein being plotted against the molarity of the sucrose gradient.

(a) Control liver. The distribution of the enzymes along the density gradient did not completely mirror that of the protein. This effect is most noticeable for cytochrome *c* oxidase, the activity being more concentrated in the less dense particles for heavy mito-

chondria, in contrast with the distribution for light mitochondria. Succinate dehydrogenase and cytochrome *c* oxidase had somewhat similar distributions for heavy and light mitochondria and, in terms of specific activity, that for cytochrome *c* oxidase of light mitochondria was 1.7 times as high as that for heavy mitochondria. For succinate dehydrogenase this difference was much less marked and the fluffy-layer activity was nearly twice that for cytochrome *c* oxidase. This observation on the succinate-dehydrogenase activity of fluffy layer agrees with those of other authors (Laird, Nygaard, Ris & Barton, 1953; Kuff & Schneider, 1954). The distribution for NAD- and NADP-isocitrate dehydrogenases of heavy mitochondria mirrored that for protein more closely. The NADP-isocitrate dehydrogenase of light mitochondria was concentrated in the less dense particles, this distribution being in contrast with that for cytochrome *c* oxidase.

(b) Regenerating liver. (i) Cytochrome *c* oxidase. In general during liver regeneration the distribution of enzyme activity followed that for protein, the light mitochondria corresponding to a density of 1.4M-sucrose increasing in amount before the heavy mitochondria. During the period of rapid mitochondrial synthesis, 15-22 days after partial hepatectomy, the heavy mitochondria corresponding to a density of 1.5M-sucrose had a low content of cytochrome *c* oxidase in contrast with a high NAD-isocitrate dehydrogenase content. The light mitochondria were considerably less heterogeneous at this point. By the 'end' of regeneration, the distribution figures resembled the control. Some heterogeneity for fluffy layer became evident during liver regeneration, in contrast with the near homogeneity of control liver. The less dense particles were considerably enriched in cytochrome *c* oxidase 10-22 days after partial hepatectomy. (ii) Succinate

Fig. 1. Effect of liver regeneration on the distribution of cytochrome c oxidase (○) and protein (●) in heavy mitochondria separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy. In this and subsequent Figures all abscissa values are those given in (g).

Fig. 2. Effect of liver regeneration on the distribution of succinate dehydrogenase (○) and protein (●) in heavy mitochondria separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 3. Effect of liver regeneration on the distribution of NAD-isocitrate dehydrogenase (○) and protein (●) in heavy mitochondria separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 4. Effect of liver regeneration on the distribution of NADP-isocitrate dehydrogenase (○) and protein (●) in heavy mitochondria separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 5. Effect of liver regeneration on the distribution of cytochrome c oxidase (○) and protein (●) in light mitochondria separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 6. Effect of liver regeneration on the distribution of succinate dehydrogenase (○) and protein (●) in light mitochondria separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 7. Effect of liver regeneration on the distribution of NAD-isocitrate dehydrogenase (○) and protein (●) in light mitochondria separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 8. Effect of liver regeneration on the distribution of NADP-isocitrate dehydrogenase (○) and protein (●) in light mitochondria separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 9. Effect of liver regeneration on the distribution of cytochrome c oxidase (○) and protein (●) in fluffy layer separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 10. Effect of liver regeneration on the distribution of succinate dehydrogenase (O) and protein (●) in fluffy layer separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 11. Effect of liver regeneration on the distribution of NAD-isocitrate dehydrogenase (○) and protein (●) in fluffy layer separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

Fig. 12. Effect of liver regeneration on the distribution of NADP-isocitrate dehydrogenase (○) and protein (●) in fluffy layer separated by a linear sucrose-density gradient. (a) Control liver; (b) 2 days; (c) 6 days; (d) 10 days; (e) 15 days; (f) 22 days; (g) 31 days after partial hepatectomy.

dehydrogenase. The distribution behaviour for heavy and light mitochondria was very similar to that of cytochrome *c* oxidase, the figures at the end of regeneration resembling those for control liver. However, the fluffy-layer behaviour was different. At 10 days after partial hepatectomy the particles corresponding to sucrose density 1.5M were considerably enriched in the enzyme, this distribution differing a little from that of cytochrome *c* oxidase. At 31 days the fluffy layer distribution resembled that for control liver. (iii) NAD–isocitrate dehydrogenase. A considerable degree of homogeneity was indicated by the distribution for this enzyme. One point is that the heavy mitochondria, apart from having the highest specific activity, were enriched at a density of 1.5M-sucrose 10–22 days after partial hepatectomy. The fluffy layer did not illustrate any particular enrichment similar to that shown by cytochrome *c* oxidase and succinate dehydrogenase during the course of liver regeneration. (iv) NADP–isocitrate dehydrogenase. The distribution for heavy mitochondria closely mirrored that for protein during regeneration, but the less dense light mitochondria were a little more enriched in the enzyme. A feature of fluffy layer behaviour 10–22 days after partial hepatectomy was a concentration in particles of similar density to cytochrome *c* oxidase and succinate dehydrogenase. The fluffy layer also possessed a high and variable specific activity, as brought out under the section on total enzymic activity.

DISCUSSION

Mitochondria and fluffy layer have been analysed by differential and density-gradient centrifugation, and several interesting features have been observed during the course of liver regeneration. Fluffy layer differed in behaviour from mitochondria and may be partly derived from a submitochondrial particle. Evidence has also been given for the development of mitochondrial and fluffy-layer activity during regeneration and for a certain degree of heterogeneity in the mitochondrial and fluffy-layer fractions as measured by enzyme and protein distribution along a density gradient.

The identity and nature of fluffy layer has been the subject of considerable discussion; Siekevitz (1952), Jackson, Walker & Pace (1953) and Novikoff, Podber, Ryan & Noe (1953) suggested fluffy layer to be a mixture of mitochondria and microsomes. This view has also been favoured by Getz, Bartley, Stirpe, Notton & Renshaw (1962) on the basis of the lipid composition of fluffy layer being intermediate between that of mitochondria and microsomes. On the other hand Kuff & Schneider (1954) and Laird *et al.* (1953) drew attention to the high succinate-dehydrogenase activity of fluffy layer; Miller, Bagot & Greenberg (1955) produced evidence from isotope-incorporation experiments that fluffy layer was distinct from the microsomal fraction and was more similar to mitochondria.

During regeneration the increase in the amount of mitochondrial protein varied inversely with that of fluffy layer, which, if it were an artifact of the homogenization process, might be expected to vary parallel with the mitochondria (that is, if fluffy layer arose as a result of mitochondrial fragmentation). There is also the possibility that independent variation of microsomal contamination could cause the apparent variation in the amount of fluffy layer.

To test this possibility assays of a ‘classical’ microsomal enzyme, glucose 6-phosphatase, were carried out (Hers, Berthet, Berthet & de Duve, 1951; for experimental details, see the Experimental section). Results are given in Table 5. Both heavy and light mitochondria had low and similar specific activities, and the fluffy-layer fraction was six times as active, and about 44% as active as the microsomal fraction. If glucose 6-phosphatase is a true microsomal enzyme, then these results would seem to indicate mitochondria as being 7%, and fluffy layer about 44%, contaminated by microsomes. Mitochondria have been shown to contain RNA (Truman & Korner, 1962; Mil’man & Kuzyaeva, 1962; Roodyn, 1962; Kroon, 1963) and it is possible that the mitochondrial glucose 6-phosphatase is associated with this. The mitochondria and fluffy layer were washed four and three times respectively by sucrose during preparation. The

Table 5. *Glucose 6-phosphatase activity of mitochondria and fluffy layer*

Experimental details are given in the text. Specific activity is expressed as μ moles of phosphate liberated/15 min./mg. of protein at 37°. Results are given as means $\pm$ S.D. from three separate experiments, livers from two rats being used in each.

	Homo- genate	Nuclear	Heavy mito- chondria	Light mito- chondria	Fluffy layer	Micro- somal fraction	Super- natant
Specific activity	1.60 $\pm$ 0.03	1.43 $\pm$ 0.11	0.38 $\pm$ 0.01	0.40 $\pm$ 0.07	2.44 $\pm$ 0.54	5.56 $\pm$ 0.54	0.13 $\pm$ 0.02
Percentage distribution of total activity	100	25.5 $\pm$ 0.6	1.9 $\pm$ 0.3	1.8 $\pm$ 0.2	7.1 $\pm$ 1.8	54.8 $\pm$ 3.1	2.6 $\pm$ 0.5
Recovery					93.7 $\pm$ 2.0		

work of Claude (1954) suggests that, on initial centrifugation, mitochondria would be about 10% contaminated by microsomes, and that each washing would cause a tenfold reduction in contamination. Theoretically, the mitochondria would be 99.99% pure, with the fluffy layer somewhat less than that since the differential in particle size between microsomes and fluffy layer is much reduced. From these arguments the true microsomal contamination of the mitochondrial and fluffy-layer fractions must be considerably less than the figures immediately suggested on the basis of the glucose 6-phosphatase assay. Furthermore, the high succinate-dehydrogenase activity of fluffy layer would support such a contention.

In the period before and after rapid mitochondrial formation there was a rise in the amount of fluffy-layer, and an explanation for this observation could be that fluffy layer contains young incomplete mitochondria and old degenerate mitochondria and thus represents a measure of both mitochondrial synthesis and breakdown. The distribution of mitochondria and fluffy-layer protein along the density gradient was similar, and the small changes in density which were observed during regeneration occurred almost simultaneously for both fractions. The enzymic studies give several relevant points: the changes during regeneration of mitochondrial and fluffy-layer enzyme content were not similar (as shown by cytochrome *c* oxidase, succinate dehydrogenase and NADP-isocitrate dehydrogenase; NAD-isocitrate dehydrogenase was an exception); fluffy layer had a high and variable succinate-dehydrogenase activity, confirming the results of Kuff & Schneider (1954), and had the same density distribution and much the same enzymic constitution as mitochondria. Fluffy layer thus appeared to behave as an independent cell fraction with much of the characteristics of mitochondria, and may well represent broken-down and partly formed mitochondria.

During the first 10 days of liver regeneration there was a progressive increase in the fluffy-layer activities of succinate dehydrogenase and NADP-isocitrate dehydrogenase. It is tempting to speculate that these increases can be related to the elaboration of mitochondrial structure, succinate dehydrogenase possibly being laid down as part of a primary lipoprotein structure. The striking activity of fluffy-layer NADP-isocitrate dehydrogenase shortly after partial hepatectomy could probably be related to NADPH production for lipid biosynthesis, and this observation might be correlated with that of Harkness (1952), who found that total liver lipid was the most rapidly restored of the liver components.

In this context it can be noted that Goebell & Klingenberg (1963*a,b*) suggest that NAD-isocitrate

dehydrogenase is more concerned with respiratory activity (a constant-proportion enzyme) whereas NADP-isocitrate dehydrogenase is variable in activity and more likely to be related to the biosynthetic activity of the cell. The ratio of the specific activities of NADP- to NAD-isocitrate dehydrogenases in normal rat-liver mitochondria in this work was 1.5. This compares with a ratio of 2.1 (Goebell & Klingenberg, 1963*a,b*). In general the total activity of both mitochondrial and fluffy-layer NAD-isocitrate dehydrogenase fell during liver regeneration, later returning to near the control value. In contrast the fluffy-layer NADP-isocitrate dehydrogenase illustrated a marked increase in specific activity during the early stages of liver regeneration. Coupled with the observation that the actual amount of fluffy layer increased nearly twofold, this would suggest a biosynthetic role for fluffy layer, NADPH production possibly being required for lipoprotein biosynthesis.

During the course of regeneration the density of the mitochondria shifted slightly together with the total enzyme distribution. The small or light mitochondria were the first to become less dense, which suggests, as would be most likely, that these would be the first-formed mitochondria. Fluffy layer gave a similar behaviour of density change, but the enzyme distribution illustrated several interesting features. The lighter particles at 10 to 22 days after partial hepatectomy showed a marked concentration of cytochrome *c* oxidase, succinate dehydrogenase and NADP-isocitrate dehydrogenase. However, the position of the peaks for cytochrome *c* oxidase and succinate dehydrogenase differed, suggesting that the structures on which these two enzymes are laid down may not be the same. If they are in some premitochondrial form they may be able to be separated partially by density-gradient centrifugation. This difference in distribution in fluffy layer during regeneration was not evident in light mitochondria to nearly the same degree. It is thus probable that on completion of the full mitochondrial structure the heterogeneity evident in fluffy layer partially disappears.

If mitochondria are formed as a result of the division of a large mitochondrion, then the products of division should possess the same composition as the 'parent' mitochondrion. There should thus be a homogeneity in density and enzymic behaviour. This appears not to be the case for fluffy layer and light mitochondria in relation to heavy mitochondria. It would appear then from this long-term study that mitochondria may be formed from pre-existing structures which may or may not arise anew. However, it should be emphasized that there may be several types of mitochondrial morphogenesis, mitochondrial division possibly being a short-term process.

The study of the four enzymes, cytochrome *c* oxidase, succinate dehydrogenase, NAD- and NADP-isocitrate dehydrogenases, has characterized mitochondria and indicates that there is a certain degree of heterogeneity according to both size and density. Light and heavy mitochondria, as well as having different specific activities for cytochrome *c* oxidase, differed also in their distribution along a density gradient both for total protein and enzymic activity. The position of the 'peak' of protein distribution for heavy mitochondria may be estimated to correspond to a density of 1.198, whereas that for light mitochondria is 1.193. A similar but less marked behaviour was shown by succinate dehydrogenase. On the other hand, very little heterogeneity was evident for NAD-isocitrate dehydrogenase in heavy and light mitochondria except that heavy mitochondria, corresponding to a sucrose density of 1.5M, exhibited some concentration 10–22 days after partial hepatectomy. It might appear then that the heavy or large mitochondria concentrate this enzyme after the basic electron-transport structural framework has been completed. Perhaps of direct relevance is the observation that the corresponding heavy mitochondria were 'depleted' of cytochrome *c* oxidase and succinate dehydrogenase. More generally, this might be interpreted in terms of Krebs-cycle enzymes being synthesized at a later stage than those involved in electron transport. In contrast with NAD-isocitrate dehydrogenase, the NADP enzyme distribution was more heterogeneous for light mitochondria, there being a slight concentration of the enzyme in the less dense particles.

Biochemical heterogeneity of mitochondria has been demonstrated in brain tissue by Tolani & Talwar (1963), who studied cytochrome *c* oxidase. In the present study no attempt was made to separate mitochondria from different regions of the liver, and consequently it is not possible to say whether the biochemical heterogeneity of the liver mitochondria is on the tissue or intracellular level. However, the demonstration of a certain heterogeneity, as illustrated by the four enzymes studied, confirms the existence of a population which is dynamic and is a reflexion of its turnover. In all probability this heterogeneity will also be on the cellular level, since there must exist a spread of mitochondria in terms of age and this spread will be reflected in a variation of mitochondrial characteristics. It would appear from these studies that the washed 'pure' mitochondrial fraction, as defined by the classical process of differential centrifugation, is not homogeneous, there being a certain degree of heterogeneity both in terms of physical properties and enzymic activity.

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