Studies on the Biosynthesis of Protein and Lipid Components of Rat Liver Mitochondria

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1. Male rats were injected intraperitoneally with L-[³⁵S]methionine, [³²P]-phosphate and [2-¹⁴C]acetate. The animals were killed at various times up to 72 hr. after injection, and liver mitochondria were prepared and fractionated into soluble protein, insoluble protein and lipid for assay of the radioactivity of each fraction. 2. The maximal specific radioactivity of total mitochondrial phospholipid with respect to both ³²P and ¹⁴C was attained after approx. 6 hr. 3. ³²P was incorporated most rapidly into phosphatidylethanolamine, maximal incorporation being attained after approx. 6 hr.; maximal incorporation into lecithin occurred after 6–12 hr. The specific radioactivity of cardiolipin was still slowly increasing at the end of the experiment (72 hr.). 4. There were no major differences between the rates of incorporation of ¹⁴C into the lecithin, phosphatidylethanolamine and cardiolipin fractions of mitochondrial phospholipid, maximal incorporation in each case occurring after approx. 6 hr. 5. Maximal incorporation of ³⁵S into both soluble and insoluble protein fractions was attained less than 12 hr. after injection, the maximal specific radioactivity of soluble protein being higher than that of insoluble protein.

Fletcher & Sanadi (1961) studied the turnover of rat-liver mitochondrial fractions. Insoluble protein, soluble protein, lipid and cytochrome c were found to have similar half-lives, and these authors suggested that mitochondria turn over as an entity with a half-life of 10.3 days. Bailey, Taylor & Bartley (1967), using similar methods, compared the half-lives of components from the liver mitochondria of normal and essential fatty acid-deficient rats. It was shown that mitochondrial lipids turn over more slowly in essential fatty acid-deficient rats than in control animals, and also that in both sets of animals both mitochondrial lipid and soluble protein appear to contain components with differing half-lives. Both fractions appeared to have components with half-lives considerably shorter than 10 days. Fletcher & Sanadi (1961) had suggested that in their experiments mitochondrial lipid might have contained a rapidly-turning-over fraction that they called 'metabolic lipid'. The work of Pascaud (1964) and Gurr, Prottey & Hawthorne (1965) also suggested that mitochondrial lipid, at least in part, turns over rapidly. Pascaud (1964) studied the incorporation of [¹⁴C]acetate into rat-liver mitochondrial lipids and concluded that mitochondrial lipids turn over with a half-life of several hours. Gurr *et al.* (1965) followed the incorporation of

[³²P]phosphate into rat-liver mitochondrial phospholipids and suggested that mitochondrial phospholipids turn over with a half-life of 19 hr.

Although a number of investigations have been carried out on the incorporation of amino acids into mitochondrial protein *in vitro*, few studies have been made *in vivo*. Fletcher & Sanadi (1961) studied the incorporation of L-[³⁵S]methionine into mitochondrial protein and suggested that maximal incorporation occurred after several days. In similar studies, Bailey *et al.* (1967) found that ³⁵S was maximally incorporated into mitochondria less than 3 days after injection of L-[³⁵S]methionine. Beattie, Basford & Koritz (1966) have shown that [¹⁴C]leucine or [¹⁴C]valine are maximally incorporated into mitochondrial protein several hours after intravenous injection into rats.

The experiments reported here were carried out in an attempt to shed more light on the problem of the biosynthesis and turnover of mitochondrial components.

MATERIALS AND METHODS

Animals. The animals used were adult male Wistar rats (each weighing 300–400 g.) obtained from 'Shell' Research Ltd., Sittingbourne, Kent. They were given Oxoid diet 86 cubes *ad libitum*.

Injection of animals and preparation of mitochondrial components. Each animal was injected intraperitoneally with 5 ml. of 0.9% (w/v) NaCl containing $16.6\mu\text{C}$ of sodium $[2\text{-}^{14}\text{C}]\text{acetate}$, $16.6\mu\text{C}$ of $\text{L-}[^{35}\text{S}]\text{methionine}$ and $144\mu\text{C}$ of sodium $[^{32}\text{P}]\text{phosphate}$. Killing of the animals and preparation of mitochondria and mitochondrial components were carried out as described by Bailey *et al.* (1967), except that cell debris and nuclei were sedimented at $600g_{av}$ for 10 min., resuspended in 4 vol. of 0.25 M-sucrose and re-sedimented at $600g_{av}$ for 10 min.

Chemicals and radiochemicals. All chemicals and solvents used were of A.R. grade and unless otherwise stated were obtained from British Drug Houses Ltd., Poole, Dorset. Sodium $[2\text{-}^{14}\text{C}]\text{acetate}$ (27.4 mc/m-mole), $\text{L-}[^{35}\text{S}]\text{methionine}$ (52 mc/m-mole) and sodium $[^{32}\text{P}]\text{phosphate}$, injection B.P., were obtained from The Radiochemical Centre, Amersham, Bucks.

Separation of phospholipids. Phospholipid was separated from neutral lipid on silicic acid columns, and then fractionated into lecithin, phosphatidylethanolamine and cardiolipin by thin-layer chromatography (Bailey *et al.* 1967). When the phospholipid composition of the total mitochondrial lipid was to be determined the following procedure was adopted. Fractionation of the total lipid was carried out by thin-layer chromatography. The phospholipid areas were located by spraying with conc. H_2SO_4 , scraped off the plate and heated with 1.0 ml. of 73% (w/v) HClO_4 until the suspension became white. The suspension was cooled, 10 ml. of water was added, and then the suspension was centrifuged at full speed for 10 min. in an M.S.E. Super Minor bench centrifuge. The supernatant fluid was decanted and phosphate was estimated by the method of King (1932).

Radioassay. Radioassay of mitochondrial components was carried out as described by Bailey *et al.* (1967).

RESULTS

Incorporation of $[^{32}\text{P}]\text{phosphate}$ into mitochondrial phospholipids. Fig. 1 shows the variation of specific radioactivity with respect to ^{32}P with time for the total mitochondrial phospholipid fraction. The phospholipid fraction attains its maximal specific radioactivity after about 6 hr. From the decline in specific radioactivity after 6 hr. it can be calculated that the half-life of the phospholipid is approx. 1.9 days, which agrees with the value of 1.6 days obtained by Bailey *et al.* (1967) for the half-life of the fast phospholipid component of control rat-liver mitochondria.

Incorporation of $[2\text{-}^{14}\text{C}]\text{acetate}$ into mitochondrial phospholipids. Fig. 1 also shows the variation of specific radioactivity with respect to ^{14}C with time for the total mitochondrial phospholipid fraction. ^{14}C is rapidly incorporated into the phospholipid fraction, maximal incorporation occurring after about 6 hr. There is a rapid fall in specific radioactivity between 6 hr. and 12 hr. and then a steady decline in specific radioactivity after 12 hr. From the rate of decline of specific radioactivity after 12 hr. it can be calculated that the half-life of the

Fig. 1. Changes in specific radioactivity of rat-liver mitochondrial phospholipid, with respect to ^{14}C and ^{32}P , at intervals after the injection of the labelled mixture. ●, ^{14}C specific radioactivity; ○, ^{32}P specific radioactivity. Each point is the mean of the results from four animals. Each rat was injected intraperitoneally with 5.0 ml. of 0.9% NaCl containing $16.6\mu\text{C}$ of sodium $[2\text{-}^{14}\text{C}]\text{acetate}$, $16.6\mu\text{C}$ of $\text{L-}[^{35}\text{S}]\text{methionine}$ and $144\mu\text{C}$ of sodium $[^{32}\text{P}]\text{phosphate}$. Batches of rats were killed at 1, 2, 6, 12, 18, 24, 36, 48 and 72 hr. after injection. Mitochondria were prepared from the liver of each animal, and from each sample soluble protein, insoluble protein and total phospholipid were separated. The specific activities of the protein fractions with respect to ^{35}S and of the phospholipid fractions with respect to ^{14}C and ^{32}P were determined. Each sample of mitochondrial phospholipid was separated into lecithin, phosphatidylethanolamine, cardiolipin and remainder fractions by thin-layer chromatography, and the distribution of ^{14}C and ^{32}P between the individual phospholipid fractions of each sample was determined.

phospholipid is approx. 1.9 days, which is the value obtained from the ^{32}P -labelling experiment. The rapid decline in specific radioactivity of the ^{14}C -labelled phospholipid between 6 hr. and 12 hr. indicates a component with a very short half-life (maximum half-life 4 hr.).

Phospholipid composition of mitochondria. The phospholipid composition of mitochondria (mean results for three animals) is as follows: the cardiolipin fraction contained 7.6%, the lecithin fraction 55.7%, the phosphatidylethanolamine fraction 28% and the remainder 8.7% of the total phospholipid P.

Distribution of ^{32}P . Fig. 2 shows the distribution at the various time-intervals of ^{32}P between the phospholipid fractions, and Fig. 3 shows the variation with time of the specific radioactivities for the cardiolipin, phosphatidylethanolamine and lecithin fractions, assuming that no change in P distribution takes place between the phospholipid fractions. From Fig. 3 it can be seen that the ^{32}P is most rapidly incorporated into the

Fig. 2. Changes in the distribution of ^{32}P between the lecithin, phosphatidylethanolamine and cardiolipin fractions of mitochondrial phospholipid at intervals after injection of the labelled mixture. Each point is the mean of the results from four animals. Δ , Lecithin; $\circ$, phosphatidylethanolamine; $\bullet$, cardiolipin. Details are as in Fig. 1.

phosphatidylethanolamine fraction, maximal incorporation being attained after approx. 6 hr. Maximal incorporation into lecithin occurs after 6–12 hr. The specific radioactivity of the cardiolipin fraction appears to be slowly increasing throughout the course of the experiment. This observation is consistent with the finding of Bailey *et al.* (1967) that cardiolipin did not acquire its maximal percentage of the total ^{32}P radioactivity until 7 days after injection. The fact that most of the ^{32}P is in the lecithin and phosphatidylethanolamine fractions at 6 hr. explains why the maximal specific radioactivity of the total phospholipid fraction is attained after approx. 6 hr. (see Fig. 1).

Distribution of ^{14}C . Fig. 4 shows the distribution at the various time-intervals of ^{14}C between the phospholipid fractions. The distribution of activity-time curves are clearly different from those obtained with ^{32}P (Fig. 2). The percentage of the total ^{14}C radioactivity present in the phosphatidylethanolamine fraction varies very little during the course of the experiment, whereas the percentage of the total ^{14}C radioactivity present in the lecithin fraction changes from 62% at 1 hr. to 43% at 72 hr. The result for the lecithin fraction is the reverse of

Fig. 3. Changes in ^{32}P specific radioactivity of mitochondrial phospholipid with time. Each point is the mean of the results from four animals. Δ , Lecithin; $\circ$, phosphatidylethanolamine; $\bullet$, cardiolipin. Details are as in Fig. 1.

Fig. 4. Changes in the distribution of ^{14}C between the lecithin, phosphatidylethanolamine and cardiolipin fractions of mitochondrial phospholipid at intervals after injection of the labelled mixture. Each point is the mean of the results from four animals. Δ , Lecithin; $\circ$, phosphatidylethanolamine; $\bullet$, cardiolipin. Details are as in Fig. 1.

that obtained with ^{32}P . The percentage of the total ^{14}C radioactivity present in the cardiolipin fraction is essentially constant throughout the experiment.

Fig. 5 shows the variation of specific radioactivity with time for the cardiolipin, phosphatidylethanolamine and lecithin fractions, which all reach their maximal specific radioactivities at approx. 6hr. The specific radioactivities fall rapidly between 6hr. and 12hr. and then fall steadily to the end of the experiment.

Incorporation of L-[³⁵S]methionine into mitochondrial protein fractions. Table 1 shows the change in the specific radioactivity of mitochondrial soluble and insoluble protein fractions with time. ³⁵S is rapidly incorporated into both protein

fractions and each fraction attains its maximal specific radioactivity less than 12hr. after the injection of L-[³⁵S]methionine. The specific radioactivity of the soluble protein fraction is 1.46 times as high as that of the insoluble protein at 1hr., but at 72hr. the fractions have similar specific activities (soluble/insoluble specific radioactivity ratio 1.12). This indicates that the soluble protein, or a component of the soluble protein, is turning over more quickly than the insoluble protein. This conclusion is consistent with the results of Bailey *et al.* (1967), who found that soluble protein contained a component with a half-life shorter than the 9-day half-life of insoluble protein.

DISCUSSION

Several attempts have been made to determine the rate of turnover of rat-liver mitochondrial lipid by measuring either the rate of incorporation of radioactive precursors into, or the rate of loss of radioactive material from, the lipid. Conflicting reports have been published, and it seems likely that the differences in lipid half-lives obtained are due to the type of radioactive material used and the time over which the incorporation or loss of radioactivity was studied.

A number of workers have labelled mitochondrial lipid by injecting rats with [¹⁴C]acetate. Pascaud (1964) studied the incorporation of [¹⁴C]acetate into mitochondrial phospholipids over a period of 6hr. and concluded that the lipids have a half-life of 3hr. The work described in the present paper, in which the incorporation of [¹⁴C]acetate has been studied over 3 days, indicates the presence of not only a component with a short half-life (4hr.) but also one with a half-life of 1.9 days. Bailey *et al.* (1967) followed the loss of ¹⁴C from mitochondrial lipid between 3 days and 35 days after injection,

Fig. 5. Changes in ¹⁴C specific radioactivity of mitochondrial phospholipid fractions with time. Each point is the mean of the results from four animals. Δ , Lecithin; $\circ$, phosphatidylethanolamine; $\bullet$, cardiolipin. Details are as in Fig. 1.

Table 1. *Changes in the specific radioactivity of mitochondrial soluble and insoluble protein fractions with time*

Rats were injected with L-[³⁵S]methionine, and the specific activities of the mitochondrial protein fractions were determined at various time-intervals after injection. Each result is the mean for four animals.

Time after injection (hr.)	Sp. radioactivity (counts/min./mg. of benzidine sulphate)		Soluble protein/insoluble protein sp. radioactivity ratio
	Soluble protein	Insoluble protein	
1	2315	1585	1.46
2	1642	1209	1.35
6	2528	1770	1.43
12	1145	992	1.16
18	1440	1260	1.14
24	1417	1278	1.14
36	1308	1119	1.17
48	658	734	0.90
72	1122	1004	1.12

and obtained results that indicated the presence of a 1–2-day half-life component and also one of very long half-life. Fletcher & Sanadi (1961) obtained a lipid half-life of 10–3 days by following the loss of ^{14}C between 14 days and 28 days after injection of [^{14}C]acetate. As Bailey *et al.* (1967) have pointed out, the ^{14}C -labelling experiments probably measure the turnover of mitochondrial fatty acids, and it seems possible that the fatty acids turn over with a half-life of 3–4 hr. or less, and that the longer half-life values obtained represent the turnover of a precursor pool of fatty acid.

Several investigators have labelled mitochondrial phospholipid by injecting rats with [^{32}P]phosphate. Gurr *et al.* (1965) studied the incorporation of [^{32}P]phosphate into mitochondrial phospholipids over a period of 18 hr. and concluded that the half-life of the lipid is about 19 hr. Similar results have been obtained in the present paper (half-life of phospholipid 1.9 days) and by Bailey *et al.* (1967), who obtained a half-life for mitochondrial phospholipid of 1.6 days. However, Bailey *et al.* (1967) also showed that mitochondrial phospholipid contained a component with a half-life of 10 days. From these results it seems probable that the acyl groups of mitochondrial phospholipid turn over more rapidly than the rest of the molecule.

The pattern of incorporation of ^{32}P into individual phospholipids was similar to that obtained by Gurr *et al.* (1965). ^{32}P was rapidly incorporated into phosphatidylethanolamine and lecithin, as would be expected if mitochondria make their own phospholipids. If mitochondrial lecithin is primarily synthesized on the microsomes, as suggested by Wilgram & Kennedy (1963), then the lecithin formed must be rapidly transferred to the mitochondria. The slow incorporation of ^{32}P into cardiolipin confirms the observations of Bailey *et al.* (1967). As these authors have pointed out, it is possible that cardiolipin is predominantly present in the phospholipid component with a half-life of 10 days.

Despite the very obvious differences between the patterns of incorporation of [^{32}P]phosphate into lecithin, phosphatidylethanolamine and cardiolipin (particularly as regards the time required for maximal incorporation of ^{32}P), the curves showing specific radioactivity of ^{14}C against time for these three phospholipids are similar. This shows that, although the glycerophosphate moiety of cardiolipin turns over very slowly compared with that of lecithin and phosphatidylethanolamine, the acyl groups of all three phospholipids turn over at a similar rapid rate.

L-[^{35}S]Methionine was rapidly incorporated into mitochondrial proteins, as were [^{14}C]valine and

[^{14}C]leucine in the experiments of Beattie *et al.* (1966). These results are contrary to those of Fletcher & Sanadi (1961), who found a lag of several days before maximal incorporation of L-[^{35}S]methionine into mitochondrial protein. Beattie *et al.* (1966) found that insoluble protein was labelled more rapidly than soluble protein and suggested that insoluble protein is formed inside the mitochondria, but soluble protein is formed outside the mitochondria. Support for this idea has come from the work of Haldar, Freeman & Work (1966). In our experiments the specific radioactivity of the soluble protein is initially higher than that of the insoluble protein. However, specific radioactivities are expressed as counts/min./mg. of benzidine sulphate and will therefore be dependent on the amount of sulphur present in each protein fraction. If the insoluble protein fraction contained more sulphur (in a form other than methionine) than the soluble protein, then it would be difficult to tell whether label was being incorporated into insoluble protein more rapidly than into soluble protein.

Results from the type of incorporation study described are difficult to interpret and it is hard to draw conclusions about turnover times from the present work. However, the results do indicate that the two groups of mitochondrial proteins separated have different half-lives and also that different parts of the phospholipid molecule are replaced at different rates. It seems likely that, if the groups of proteins measured were separated into their individual components, then these would also be found to turn over at different rates. Similarly it is possible that, within a phospholipid fraction, compounds with different fatty acid compositions have different half-lives.

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