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Turnover of Mitochondrial Components of Normal and Essential Fatty Acid-Deficient Rats

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1. Essential fatty acid (EFA)-deficient and control rats were injected intraperitoneally with [^{32}P]phosphate, L-[^{35}S]methionine and [2-^{14}C]acetate. The animals were killed at various time-intervals after injection and their liver mitochondria fractionated into soluble protein, insoluble protein, and lipid. 2. The ^{35}S was assayed in the protein fractions and ^{32}P and ^{14}C were assayed in the lipid fraction. Curves of log (specific activity) plotted against time were prepared for the different fractions. 3. There was no significant difference between the insoluble protein results for control and EFA-deficient animals, both sets of results indicating the presence of a single component of half-life 9 days. 4. There was no significant difference between the soluble protein results for the two sets of animals and both sets of results indicated the presence of at least two components. 5. The [^{32}P]-phospholipid results indicate that in the control animals the liver mitochondrial phospholipids contain components of half-life 1·6 and 10 days whereas the mitochondrial phospholipids of the EFA-deficient animals contain components of half-life 3 and 29 days. 6. The specific activity of mitochondrial [^{14}C]phospholipid initially fell rapidly in both groups of animals, but after 17 days there was no further significant decrease. A fast component with maximum half-life 2–4 days was clearly demonstrated for both groups of animals. Whether or not these results also indicate the presence of a very long-lived mitochondrial phospholipid is discussed.

Mitochondria prepared from the livers of rats deficient in EFA* differ from normal mitochondria in several respects. They swell more rapidly (Johnson, 1963*a*,*b*), they are more permeable to substrates (Hayashida & Portman, 1963) and oxidative phosphorylation in them rapidly becomes uncoupled (Johnson, 1963*b*). At the same time the dietary deficiency of EFA causes marked changes in the fatty acid composition of mitochondrial lipid (Bartley, 1964), most of which is phospholipid and thought to be predominantly structural in function. In addition, changes in mitochondrial structure have been reported in EFA deficiency (Wilson & Leduc, 1963; Stein & Stein, 1964). It was therefore decided to compare the turnover of several components of mitochondria from the livers of deficient and control rats to determine whether or not deficiency induced changes in stability either in the mitochondrion as a whole or in its chemical constituents. This study also afforded an opportunity of testing more fully the suggestion of Fletcher & Sanadi (1961) that mitochondria turn over as an entity.

* Abbreviation: EFA, essential fatty acids.

MATERIALS AND METHODS

Chemicals. Except where otherwise stated, all chemicals and solvents were of analytical grade and obtained from British Drug Houses Ltd. (Poole, Dorset). The following, used as standards in gas–liquid chromatography and thin-layer chromatography, were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.): L-α-lecithin (synthetic grade II), $\beta\gamma$-dipalmitoyl-L-α-phosphatidylethanolamine (synthetic grade II) and the methyl esters of palmitic acid (99+%), palmitoleic acid (99+%), stearic acid (99+%), oleic acid (99+%), linoleic acid (99+%), arachidonic acid (90–95%) and docosahexaenoic acid (90+%, grade II).

L-[^{35}S]Methionine (52 mc/m-mole), sodium [2-^{14}C]acetate (27·4 mc/m-mole) and sodium [^{32}P]phosphate (Injection B.P.), were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals and diet. Male rats (specific-pathogen-free) of Wistar stock, supplied by 'Shell' Research Ltd. (Sittingbourne, Kent) were put on the experimental diets 2 weeks after weaning, at which time average body weight was 75 g. Thirty-six rats were fed *ad libitum* on a synthetic diet of the

following composition: 20 parts of fat-free casein (British Drug Houses Ltd.), 70 parts of household sugar (Tate and Lyle Ltd., Liverpool) and 5 parts of salt mixture. This salt mixture consisted of salts in the following proportions by weight: NaCl, 100; $MgSO_4,7H_2O$, 315; $NaH_2PO_4,2H_2O$, 200; K_2HPO_4, 552; ferric citrate pentahydrate, 67; calcium lactate pentahydrate, 750; $CaH_4(PO_4)_2,H_2O$, 312; KI, 0·2; $CuSO_4,5H_2O$, 2·0; $MnCl_2,4H_2O$, 5·0. Vitamins were added to the diet to give the following concentrations (mg./kg. of diet): thiamine hydrochloride, 2·0; riboflavine, 4·0; nicotinamide, 10·0; inositol, 50·0; choline chloride, 2000; biotin, 0·043; pyridoxine hydrochloride, 4·0; *p*-aminobenzoic acid, 25·0; calcium pantothenate, 24·0; vitamin A acetate, 7·5; DL-α-tocopherol, 50·0; calciferol, 0·04. The last three were added in tributyrin and the others in aqueous solution. In practice large batches of diet minus vitamins were stored dry and on feeding days (three times a week) the appropriate quantity of vitamin solution was added to the portion of diet required. After the addition of approx. 15% (w/w) of water, the whole diet was mixed in a mechanical mixer. Thirty-six control rats were given a diet prepared in the same way but containing in addition 10ml. of corn oil (Mazola; supplied by Brown and Polson Ltd., Esher, Surrey) /kg. dry wt. of diet.

The rats were caged separately and weighed weekly. No deaths occurred during the experiment.

Injection and killing of animals. After 15 weeks on a diet, each deficient and control animal was injected intraperitoneally with 5·0ml. of 0·9% NaCl containing 367 μc of sodium [^{32}P]phosphate, 27·5 μc of L-[^{35}S]methionine and 27·5 μc of sodium [2-^{14}C]acetate. The rats were killed by stunning and decapitation in groups of six (three deficient and three controls) at intervals up to 5 weeks after injection. The livers were removed immediately after death, placed on ice and the mitochondria isolated as soon as possible.

Isolation of mitochondria. A 10g. sample of liver, or the whole liver if less than 10g., was pulped in a Fischer mincer and homogenized in a stainless-steel Potter–Elvehjem homogenizer at top speed for 1min. with 4vol. of ice-cold 0·25M-sucrose (pH7·4). Nuclei and cell debris were sedimented by centrifugation at 450$g_{av.}$ for 10min. The sediment was resuspended in 2·5vol. of ice-cold 0·25M-sucrose and centrifuged at 400$g_{av.}$ for 10min. The mitochondria were sedimented by centrifugation of the combined supernatant fluids (from the two previous centrifugations) at 8500$g_{av.}$ for 10min. The mitochondrial pellet was washed twice by resuspending it in 2·5vol. of ice-cold 0·25M-sucrose and sedimenting again by centrifugation at 8500$g_{av.}$ for 10min.

Separation of mitochondrial fractions. This was carried out by a modification of the method of Fletcher & Sanadi (1961). The mitochondria were suspended in 2·5vol. of ice-cold distilled water and stood on ice for 30min. The supernatant fluid, after centrifugation at 15000$g_{av.}$ for 10min., was designated 'soluble protein fraction'. Cytochrome *c* and additional soluble protein were extracted by suspending the sediment in 2·5vol. of ice-cold 0·15M-NaCl for 15min. followed by centrifugation at 15000$g_{av.}$ for 10min. (this fraction was not used for the present experiments). The sediment consisted of insoluble protein and lipid.

Lipid was removed from the residue by extracting three times with 5·0ml. of methanol–$CHCl_3$ (3:1, v/v) at 60° for 15min. The extracts were filtered through a fat-free filter paper. The filter paper itself was then extracted with 10ml. of $CHCl_3$–methanol mixture at 60° for 15min. and this extract added to the filtrate. The combined extract was evaporated to dryness at 40° in a rotary evaporator. The crude lipid was redissolved in 10ml. of $CHCl_3$–methanol (2:1, v/v) and purified by the method of Folch, Lees & Sloane-Stanley (1957). The samples of purified lipid were stored at −18° in 5ml. of $CHCl_3$. The residue from this extraction was designated 'insoluble protein'.

Measurement of specific activity of ^{35}S in the protein fractions. The soluble protein fraction was treated with an equal volume of 10% (w/v) trichloroacetic acid and after standing for 30min. on ice the precipitate was sedimented by centrifugation (maximum speed in M.S.E. Major bench centrifuge). The insoluble protein and the trichloroacetic acid-precipitated soluble protein were subjected to combustion and the resultant sulphate was precipitated as benzidine sulphate by the method of Gaitonde & Richter (1955) with the following modifications. After the residues from combustion had been dissolved in 2·0ml of N-HCl, and the volume reduced by heating to about 1ml., 2N-NaOH was added until the mixture was alkaline to pH paper. Then 30min. later the mixture was filtered through a Whatman no. 42 filter paper and the filtrate adjusted to below pH4 with 6N-HCl; 1 drop usually sufficed. The precipitation of benzidine sulphate and its deposition as an even plate on filter paper (1 in. diam., Whatman no. 42) was essentially as described by Gaitonde & Richter (1955). The plates were counted under an end-window Geiger–Müller tube connected to a Panax D657 counter. Counts were corrected to infinite thinness after allowing for dead time, background and radioactive decay. In addition, although most of the contaminating ^{32}P was removed at the alkaline precipitation stage after combustion, it was found necessary to correct for residual ^{32}P contamination. This was done by counting all samples with and without a 100mg./cm.^{2} bronze filter, which stops 99% of the radiation from ^{35}S but only 35% of that from ^{32}P. From this the contribution of ^{32}P to the unscreened count could readily be calculated. The correction rarely amounted to more than 10%.

Separation and radioactive assay of phospholipids. Total lipid extracts were applied to 6cm. × 1cm. columns of silicic acid (325 mesh for lipid chromatography; Koch–Light Laboratories Ltd., Colnbrook, Bucks.). Neutral lipids were eluted with 50ml. of $CHCl_3$ and phospholipids with 100ml. of methanol. The phosphorus content of the phospholipid fraction was determined by the method of Berenblum & Chain (1938) as modified by Bartley (1953). A portion of the extract was placed in a counting vial, evaporated to dryness under nitrogen and dissolved in NE213 liquid scintillator [Nuclear Enterprises (G.B.) Ltd. (Sighthill, Edinburgh)]. Activities of ^{32}P and ^{14}C were determined by a channels-ratio method in a Nuclear–Chicago 725 liquid-scintillation counter. The samples were counted again after 8 weeks. From the loss of activity and known decay of ^{32}P, the ^{14}C radioactivity of the samples could be calculated. The results were in agreement with those obtained from the channels-ratio method. Total phospholipid was separated into the individual phospholipids by thin-layer chromatography by the method of Wagner, Hörhammer & Wolff (1961). Radioactive areas on the chromatograms were detected by radioautography, removed from the plates, suspended in NE213 liquid scintillator by the addition of Cab-O-Sil and counted in the Nuclear–Chicago 725 liquid-scintillation counter.

RESULTS

EFA deficiency. At the start of the turnover study, i.e. after the animals had been on a diet for 15 weeks, the group given the fat-free diet showed the typical signs of EFA deficiency. They had grown less rapidly than the controls and after 12 weeks their body weight had reached a plateau, whereas the supplemented rats continued to grow. At 15 weeks the average weight of the deficient rats was 286g. and that of the supplemented rats was 378g. The skin of the deficient rats was scaly (this was particularly noticeable on the feet) and the water consumption of these animals was 70% higher than that of the supplemented rats. Table 1 shows a fatty acid analysis of the total lipid of liver mitochondria from deficient and supplemented animals. Most noticeable is the absence of linoleic acid from the deficient liver and the low percentage of arachidonic acid. Differences are not apparent in the percentages of docosahexaenoic acid, probably because corn oil used as supplement in the control series contains little linolenic acid, the precursor of docosahexaenoic acid. Clearly the rats on the fat-free diet were deficient in EFA.

Table 1. *Fatty acid composition of mitochondrial lipid from livers of EFA-deficient and control rats.*

The fatty acids are represented by $C_{x:n}$ where x is the number of carbon atoms and n is the number of double bonds.

	% of total fatty acid	
Fatty acid	Deficient	Control
C_{14}	0·6	0·4
C_{16}	22·0	21·5
$C_{16:1}$	7·7	7·1
C_{18}	15·7	21·6
$C_{18:1}$	30·4	17·6
$C_{18:2}$	0·0	7·2
$C_{20:3}$	17·7	3·2
$C_{20:4}$	4·7	19·0
$C_{22:4}$	0·2	1·2
$C_{22:6}$	1·0	1·2

Specific activity of protein fractions. Fig. 1 shows the change with time in the specific activity of sulphur from mitochondrial insoluble protein. Firstly, it is clear that there is no significant difference between the values for deficient and supplemented animals. Secondly, the fall in the logarithm of specific activity is linear with time, i.e. the decay indicates a first-order process with a half-life of 9·0 days.

Fig. 2 shows the corresponding values for mitochondrial soluble protein. Again there is no difference between the values for deficient and control rats. However, in this case the logarithmic plot of decay is not linear but a continuous curve, indicating

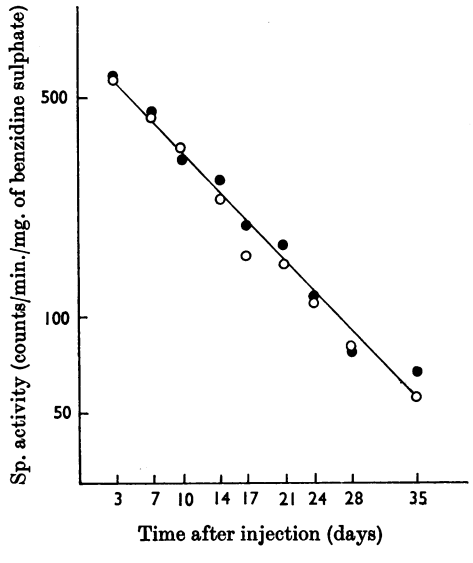

Fig. 1. Change in the logarithm of mitochondrial insoluble-protein specific activity with time. The details are as given in the text. Each point is the mean result for three animals. ●, Control rats; ○, EFA-deficient rats.

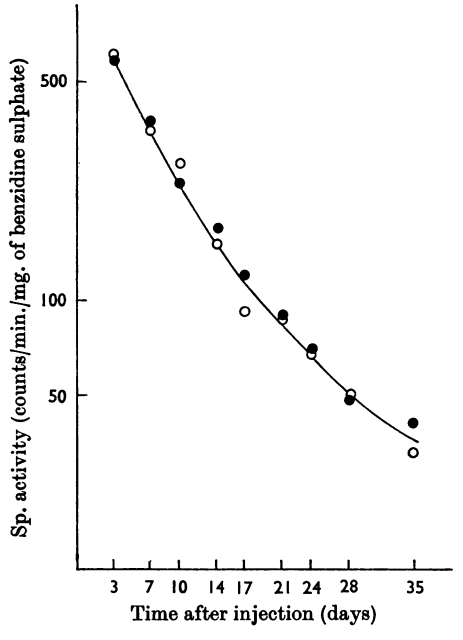

Fig. 2. Change in the logarithm of mitochondrial soluble-protein specific activity with time. The details are as given in the text. Each point is the mean result for three animals. ●, Control rats; ○, EFA-deficient rats.

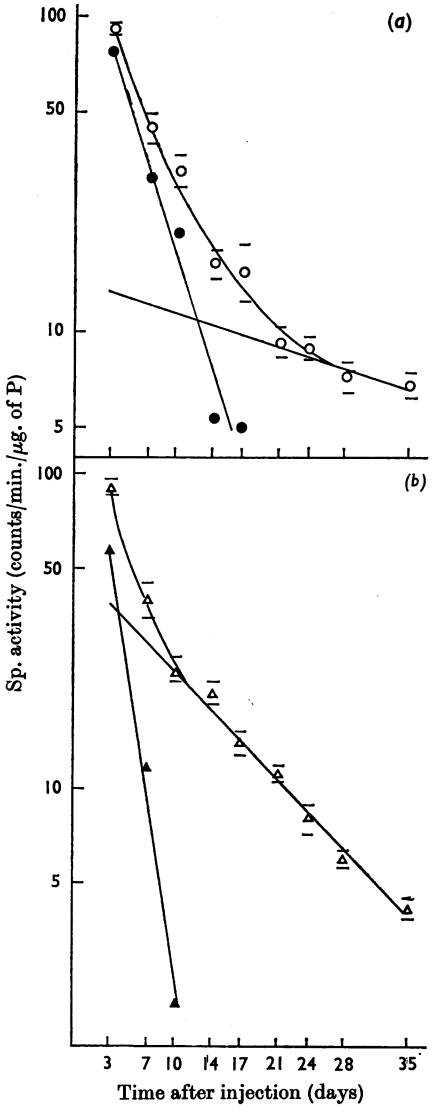

Fig. 3. Change in logarithm of mitochondrial [^{32}P]phospholipid specific activity with time. The details are as given in the text. Each point is the mean result for three animals. The horizontal bars represent the range of results. The points for the fast components were obtained by subtracting the values deduced from the extrapolation of the linear 'tail' of the experimental graph from the initial experimental points. (*a*) EFA-deficient rats; ○, experimental results; ●, calculated fast component; (*b*) control rats; △, experimental results; ▲, calculated fast component.

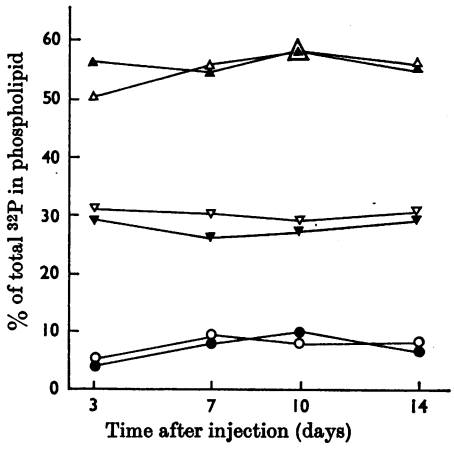

Fig. 4. Change in the distribution of total mitochondrial phospholipid ^{32}P between lecithin, phosphatidylethanolamine and cardiolipin. The details are as given in the text. Each point is the mean result for three animals. △, Lecithin of control rats; ▲, lecithin of EFA-deficient rats; ▽, phosphatidylethanolamine of control rats; ▼, phosphatidylethanolamine of EFA-deficient rats; ○, cardiolipin of control rats; ●, cardiolipin of EFA-deficient rats.

the presence of more than one component. Such a curve can be fitted on the assumption that it consists of two components decaying by first-order kinetics. In the absence of a linear 'tail' it is not possible to assign values to the half-lives of these components with any precision. The half-life calculated for the period 14–28 days (that studied by Fletcher & Sanadi, 1961) is 9·5 days, but consideration of the whole curve suggests the presence of components of shorter and longer half-life than this.

Fig. 3 shows the logarithmic decay curves for ^{32}P of mitochondrial phospholipid for deficient and control animals. In neither case is decay linear with time. The remarks on the analysis of the curves for soluble protein apply equally here, but, since curves for phospholipid for both deficient and supplemented animals are linear after 14 days, characterization of the curves in terms of two components seems justified. Treated in this way the curves yield components of 1·6 and 10 days for the supplemented rats with 3 and 29 days for deficient rats.

The variation in the proportion of total ^{32}P radioactivity of phospholipid contributed by lecithin, phosphatidylethanolamine and cardiolipin during the period 3–14 days after injection is shown in Fig. 4. The only notable change during this time is the rise in the proportion accounted for by cardiolipin from 5% at 3 days to 9–10% at 7 days. Again there is no apparent difference between results for deficient and control animals. Thus, although between 3 and 14 days the specific activity of the phospholipid falls by about 65%, the relative specific activities of lecithin and phosphatidylethanolamine are not markedly altered, indicating

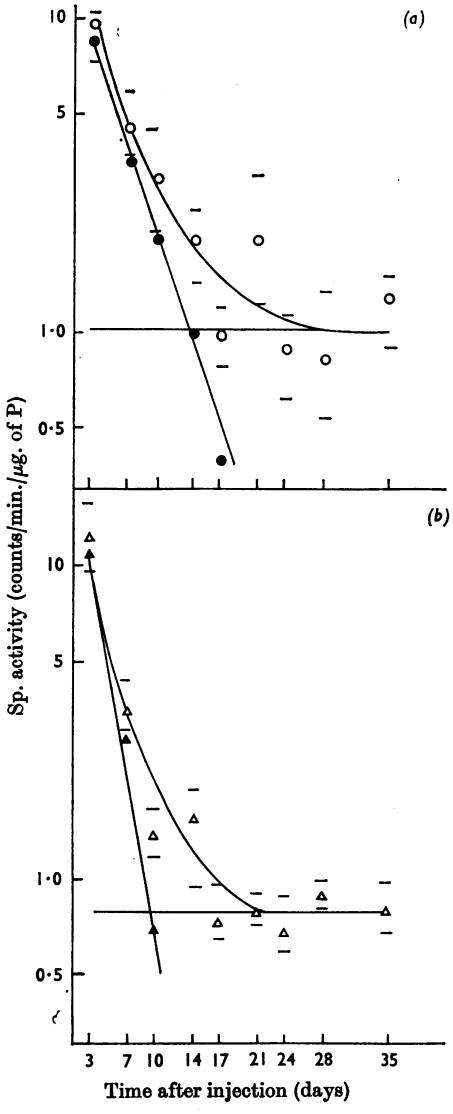

Fig. 5. Change in logarithm of mitochondrial [^{14}C]phospholipid specific activity with time. The details are as given in the text. Each point is the mean result for three animals. The horizontal bars represent the range of results. The points for the fast component were obtained by subtracting the values deduced from the extrapolation of the linear 'tail' of the experimental graph from the initial experimental points. (*a*), EFA-deficient rats; ○, experimental results; ●, calculated fast component; (*b*) control rats; △, experimental results; ▲, calculated fast component.

that during this period these two phospholipids turn over at similar rates. This conclusion applies to both deficient and supplemented groups, although the absolute rates of turnover in the groups are different.

The finding that the proportion of ^{32}P radioactivity contributed by cardiolipin increases from 5 to 10% between 3 and 7 days is in agreement with the trend noted in short-term experiments (Taylor, Bailey & Bartley, 1967). This rise indicates that the specific activity of cardiolipin is declining less rapidly than that of lecithin or phosphatidylethanolamine and may mean either that there is a very slow incorporation step or that the short-lived phospholipid component deduced from analysis of the curve for total phospholipid is poor in cardiolipin.

Fig. 5 shows the decay of ^{14}C of mitochondrial phospholipid. Initially, activity declines rapidly but the rate of fall decreases until at about 17 days the specific activity is, within experimental limits, static. Subtraction of the static component gives a fast component of half-life 2 days for the supplemented rats and 3·5 days for the deficient rats.

DISCUSSION

The present results suggest that EFA deficiency decreases the turnover of the phospholipid fraction of rat liver mitochondria. Although the effect of EFA deficiency on mitochondrial phospholipid turnover has not been reported previously, there have been several conflicting accounts of its effect on phospholipid turnover in whole liver. Klein & Johnson (1954) failed to detect any difference between the incorporation of [^{32}P]phosphate into liver phospholipids of rats given a fat-free diet and those given the same diet supplemented with 5% of corn oil. Artom, Sarzana & Segré (1938) had previously shown that the turnover of hepatic phospholipids was faster in rats given a diet rich in olive oil than in those given a fat-free diet. On the other hand, Collins (1962) found that more [^{32}P]-phosphate was incorporated into liver phospholipids by EFA-deficient rats than by control animals. A similar observation was made by Morin & Alfin-Slater (1964).

In all these investigations the radioactivities of liver phospholipids of EFA-deficient and control rats were compared at only one time-interval after injection of [^{32}P]phosphate [1hr. by Collins (1962); 4hr. by Klein & Johnson (1954) and Morin & Alfin-Slater (1964); 4 days by Artom *et al.* (1938)]. Thompson & Ballou (1954, 1956) and Pascaud (1964) have shown that there are in liver a number of lipid pools with widely differing turnover rates. It is possible that the workers comparing phospholipid radioactivities a few hours after [^{32}P]phosphate injection were in fact measuring different metabolic pools of phospholipid from Artom *et al.* (1938) 4 days after [^{32}P]phosphate injection. Thus EFA deficiency may have opposite effects on the turnover of different metabolic pools of phospholipids. Our results show that mitochondria

contain at least two phospholipid components, the turnovers of which are decreased in EFA deficiency, and it is possible that we have failed to detect components of very short or very long half-life.

The finding that mitochondrial phospholipid turnover is affected by EFA deficiency whereas mitochondrial protein turnover is unaffected is consistent with the observations of De Pury & Collins (1965), who found that deficiency does not affect protein synthesis in whole livers. It therefore seems likely that any changes in mitochondrial structure that take place in EFA deficiency, such as those reported by Wilson & Leduc (1963) and Stein & Stein (1964), are due to changes in the lipid portion of the mitochondria. However, we were unable to detect marked changes in rat liver mitochondrial ultrastructure in EFA deficiency (unpublished work).

It was the original purpose of the present experiments to compare the half-lives of mitochondria from EFA-deficient and control rats by the method of Fletcher & Sanadi (1961). These authors found that several mitochondrial fractions had similar half-lives and concluded that mitochondria turn over as an entity with a half-life of 10·3 days. Some of our findings appear to be at variance with these conclusions. First, although we find that insoluble protein has a half-life similar to that reported by Fletcher & Sanadi (1961), our soluble protein fraction appears to contain at least two components, one of which is likely to have a half-life considerably longer than 10 days. Secondly, our phospholipid results indicate that the turnovers of the two components are both lowered in EFA deficiency without a concomitant change in the turnover of the protein fractions. These results suggest that mitochondrial soluble protein, insoluble protein and phospholipid fractions turn over independently of each other.

It was assumed throughout the present study that the cell fractionation procedure used yielded comparable mitochondrial fractions for EFA-deficient and control rats. Moreover, since the mitochondrial fractions were not tested for purity it is possible that the fast components of the lipid and soluble protein fractions were derived from a non-mitochondrial contaminant. However, this possibility seems unlikely since our soluble protein and control lipid results are very similar to those obtained by Lusena & Depocas (1966), who used mitochondria prepared by a different procedure from that used by us. Further, Gurr, Prottey & Hawthorne (1965), using yet another mitochondrial preparation procedure, demonstrated the presence of a phospholipid component with a half-life very similar to the fast phospholipid component of our control mitochondria.

Apparent discrepancies between the results of Fletcher & Sanadi (1961) and our own may be due to differences in duration of the sampling period. Their conclusions are drawn from the coincidental linearity of decay in specific activity of several components, measured at 14, 21 and 28 days after injection. Our experiments, in which specific activities were measured at nine points between 3 and 35 days, agree with those of Fletcher & Sanadi (1961) over the period for which they can be compared (14–28 days), but a consideration of the data obtained before and after this period leads to the conclusion that the similarity in radioactive decay rates of the components is fortuitous. In particular, it is well established here, and also by Lusena & Depocas (1966), that the specific activity of [^{14}C]-lipid decays rapidly before this period and very slowly thereafter, so that the mean decay rate between 14 and 28 days cannot be used as a measure of lipid turnover.

The only other study comparable with ours is that of Lusena & Depocas (1966), who measured changes in specific activity of mitochondrial components 1–17 days after injection with [^{14}C]acetate. They also found that for the period 14–28 days their results agreed with those of Fletcher & Sanadi (1961) in that the mean half-lives of the several components were all about 10 days, but that for soluble protein, insoluble protein and also lipid the logarithmic plots all gave curves of continuously decreasing slope. They concluded that decay of mitochondrial components was more complex than had previously been indicated. In the early part of the decay curves obtained by Lusena & Depocas (1966) ^{14}C is lost from both soluble and insoluble protein with a half-life of about 3–5 days, clearly indicating the presence of a fast protein component.

The experiments of Wilson & Dove (1965) and Khan & Wilson (1965) appear to support the suggestion that mitochondria turn over as an entity. These workers obtained linear logarithmic decay curves for brain, liver and kidney mitochondrial protein labelled with tritium. Although these results are consistent with a single component, they may also mask a multi-component system. Unfortunately, turnover of mitochondrial lipid was not investigated in any of the tritium-labelling experiments.

A number of other reports suggest that mitochondrial components turn over independently. The work of Pascaud (1964), Gurr *et al.* (1965) and Taylor *et al.* (1967) suggests that mitochondria contain a number of lipid components with different half-lives. The experiments of Beattie, Basford & Koritz (1966) and Haldar, Freeman & Work (1966) indicate that mitochondrial soluble protein is made outside the mitochondria, whereas Roodyn, Reis & Work (1961) have shown that insoluble protein can be synthesized by the mitochondria. Droz &

Bergeron (1965) found a Poisson distribution of liver mitochondrial grain count after injecting rats with tritiated amino acids, indicating that mitochondrial protein synthesis was not confined to a small proportion of mitochondria. These authors argued that, if mitochondria turn over as an entity, incorporation of amino acids would be restricted to the 'period de genèse', which they assumed to be very short and hence only a small proportion of mitochondria should have been labelled.

In our experiments the specific activity of mitochondrial [^{14}C]phospholipid did not decrease detectably after 17 days. This could be interpreted as indicating that some mitochondrial acyl groups (assuming that [^{14}C]acetate labels predominantly the fatty acid portion of phospholipids) have a very long half-life. The specific activity of [^{14}C]lipid from heart, kidney and adipose tissue also remained essentially static after 17 days (unpublished work). However, the work of Pascaud (1964) and Taylor *et al.* (1967) suggests that at least a portion of mitochondrial fatty acids turns over very rapidly. It seems possible that kidney and heart fatty acids and liver mitochondrial fatty acids turn over very rapidly and are in equilibrium with a circulating pool of fatty acids, which is itself in equilibrium with adipose-tissue fatty acids. Stein & Stein (1962) have shown that some adipose tissue fatty acids turn over very slowly (half-lives 163–187 days). Hence, when measuring lipid turnover of tissues and organelles by [^{14}C]acetate-labelling techniques one may in fact be measuring the turnover of a precursor pool of fatty acids. This point appears to have been overlooked by other workers.

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