apparently influenced by the calcium content of the diet and it would appear likely that in animals maintained on a diet with fairly high calcium, such as the rats used in the present studies, this process might be only partly responsible for the preferential utilization of calcium over strontium.

## SUMMARY

1. The absorption of calcium, strontium, barium and radium from the gastrointestinal tract of the rat has been studied by means of double radioactive-tracer techniques.

2. At 14–18 days of age there is almost complete absorption of calcium and strontium and about 80% of barium and radium is absorbed. For animals 6–8-weeks old absorption of calcium has decreased to about 60%, strontium to 25%, barium to 7% and radium to 11%. In animals at 60–70 weeks of age the absorption of all the elements except barium is decreased to between one-third and one-half of these levels.

3. Deprivation of food before administration markedly increases the absorption of barium and radium and slightly reduces calcium absorption.

4. Administration of the elements in cow's milk significantly enhances the absorption of calcium and, more particularly, strontium in 6–8-week-old rats. The absorption of barium and radium is not affected and the effect is not observed in old animals.

5. The results for the absorption of barium and radium show a closer similarity to each other than to the results for strontium and calcium.

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# Protein Synthesis in Mitochondria

## 2*. RATE OF INCORPORATION *IN VITRO* OF RADIOACTIVE AMINO ACIDS INTO SOLUBLE PROTEINS IN THE MITOCHONDRIAL FRACTION, INCLUDING CATALASE, MALIC DEHYDROGENASE AND CYTOCHROME *c*

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It has been demonstrated previously that, under suitable conditions, isolated rat-liver mitochondria can incorporate radioactive amino acids into their proteins (Reis, Coote & Work, 1959*a*). In a more detailed study of the system (Roodyn, Reis & Work, 1961*a*) it was shown that the incorporation was not due to bacterial or microsomal contamination, was fully energy-dependent, and was very sensitive to incubation in the absence of an oxidizable substrate. The incorporation was not due to adsorption on the mitochondrial protein, or to the

* Part 1: Roodyn, Reis & Work (1961*a*).

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formation of S–S bonds. Treatment of the total mitochondrial protein with 1-fluoro-2,4-dinitrobenzene showed that only a small proportion of the total radioactive amino acids was present as *N*-terminal residues. Also, it has recently been shown that only a small proportion of the radioactivity is found in the *C*-terminal residues (J. W. Suttie, in preparation). From these results it seemed likely that the radioactive amino acids were incorporated into the body of the peptide chain and that the incorporation process was a measure of true protein synthesis in mitochondria and not an artifact in labelling.

However, conclusive proof of this required the isolation of a well characterized protein and the demonstration of the appearance in it of radioactive amino acids bound by the peptide link. Experiments were therefore carried out to determine the rate of labelling of various mitochondrial proteins. The first results with catalase were disappointing (Reis, Coote & Work, 1959*b*; Roodyn, Reis & Work, 1961*b*) and were in marked contrast with the high rate of labelling of cytochrome *c* observed by Bates, Craddock & Simpson (1958, 1960) for rat-liver mitochondria, and by Bates & Simpson (1959) for calf-heart sarcosomes. However, study of the distribution of radioactivity within various mitochondrial-protein fractions revealed that the soluble proteins, either escaping into the medium or released by disruption of the mitochondria with neutral detergents, were in general very poorly labelled. The major site of incorporation was a fraction sedimenting at 105 000*g*, rich in succinoxidase, phospholipid and RNA, and probably representing the mitochondrial membrane (Roodyn *et al.* 1961*a*; Roodyn, 1961). Preliminary chromatography of the soluble-protein fraction on calcium phosphate columns failed to reveal any proteins of high specific radioactivity, or even of the same specific radioactivity as the total mitochondrial protein. As a result of these indications, the rate of labelling of specific soluble mitochondrial proteins was examined and it was found that neither cytochrome *c* nor malic dehydrogenase was synthesized to a significant extent under the conditions used. The negative results previously obtained with catalase are also presented below.

## METHODS AND MATERIALS

### *Preparation of cell fractions*

Rat-liver mitochondria and other cell fractions were isolated as described previously (Roodyn *et al.* 1961*a*). Mitochondrial subfractions were prepared by suspending mitochondria in 0·3 M-sucrose, adding the detergent Triton X 100 to a final concentration of 0·2% and, after 10 min. at 0°, centrifuging first at 10 000*g* for 30 min. and then at 105 000*g* for 60 min.

### *Incubation with radioactive amino acids*

Mitochondria were incubated at 30° under oxygen as described previously. 'Medium B' of the above paper was used in all incubations [0·1 M-sucrose, 0·04 M-KCl, 1·3 mM-EDTA (disodium salt), 0·02 M-nicotinamide, 0·01 M-potassium succinate, 0·016 M-potassium phosphate, 4 mM-AMP, 0·5 mM-DPN, 8 mM-$MgSO_4$, 50 $\mu$g. of synthetic amino acid mixture/ml. and 3–5 mg. of mitochondrial protein/ml.]. The pH of the reaction mixture was between 7·0 and 7·2. In some experiments the medium was supplemented with 100 units of penicillin G and 60 $\mu$g. of streptomycin sulphate/ml., as indicated in the footnotes to the Tables and Figures. Uniformly $^{14}$C-labelled algal-protein hydrolysate (1 mc/5·6 mg.), prepared by the method of Simkin (1958), and L-[$^{14}$C]valine (1 mc/17·9 mg.) were used as radioactive tracers.

### *Chromatography of soluble proteins on calcium phosphate*

Soluble mitochondrial proteins were dialysed against 0·02 M-sodium phosphate, pH 7·0, and loaded by gravity on columns of calcium phosphate (brushite) prepared as described by Faulkner, Martin, Sved, Valentine & Work (1961). The proteins were eluted either by stepwise increases in phosphate buffer concentration (Tables 1 and 4) or by non-linear gradient elution, with a constant-volume mixing chamber (Fig. 2). A 50 ml. conical flask was connected to the head of the column and contained dilute buffer. Concentrated buffer flowed in from a main reservoir and was mixed by a magnetic stirrer.

### *Isolation of cytochrome* c (*Table* 2)

Mitochondria were incubated in the presence of radioactive amino acid and the reaction was stopped by cooling the suspension to 0° and adding 1·0 ml. of carrier (10 mg. of [$^{12}$C]valine/ml. or 150 mg. of casein hydrolysate/ml.) per 30 ml. of suspension. In Expt. 1, the mitochondria were centrifuged down and the pellet was extracted with sulphuric acid according to the method of Rosenthal & Drabkin (1943). The radioactivity found in the cytochrome *c* was very low, and it was thought possible that this was due to loss of cytochrome *c* into the incubation medium (cf. Dianzani & Viti, 1955). Accordingly, the method was slightly modified as follows in order to isolate cytochrome *c* simultaneously from both medium and mitochondria (Expts. 2 and 3): the entire suspension, without centrifuging down the mitochondria, was treated at room temperature with 5 ml. of 2·5 N-$H_2SO_4$ followed by 1·8 ml. of aq. 5·0 N-$NH_3$ per 30 ml. of suspension. The sulphuric acid and ammonia were added dropwise while the solution was being rapidly stirred. After 15 min. at room temperature the precipitate was removed by centrifuging at 10 000*g* for 5 min. and the supernatant adjusted to pH 7·0 with potassium hydroxide. The cytochrome *c* was isolated from the extract essentially according to the method of Keilin & Hartree (1937). Ammonium sulphate (50 g./100 ml. of solution) was added and after 15 min. at 0° the precipitate was removed by filtration through Whatman no. 1 paper. Ammonium sulphate (5 g./100 ml. of filtrate) was added, and in Expts. 1 and 3 the solution was left overnight at 0°. In Expt. 2 the solution was left for only 30 min. at 0°. In all

experiments only a very faint precipitate appeared at this stage, and was removed by filtration through Whatman no. 1 paper. To the filtrate was added 0·025 vol. of 20% (w/v) trichloroacetic acid, and after 45 min. at 0° the cytochrome *c* that separated out was centrifuged down at 3000*g* for 20 min. In Expt. 1, this was plated directly without further treatment. In Expt. 2 it was taken up in 2·0 ml. of water and loaded on a 1·0 cm. × 1·5 cm. column of ion-exchange resin CG-50 that had been previously treated according to the method of Palêus & Neilands (1950). The column was washed with 0·1 M-ammonium acetate, pH 8·9, and the cytochrome *c* was eluted with 0·5% aq. ammonia. It was precipitated with 5% trichloroacetic acid, washed once with acetone, then with ether, and plated. In Expt. 3 the cytochrome *c*, precipitated by the method of Keilin & Hartree (1937), was not further purified on the column, but was washed more thoroughly before plating than in Expts. 1 and 2. The cytochrome *c* was taken up in water, carrier amino acid added, and precipitated and washed once with 5% trichloroacetic acid. The precipitate was taken up a second time in water, more carrier added, and then reprecipitated with trichloroacetic acid. After the second washing with trichloroacetic acid the protein became no longer soluble in water, or even in aq. 0·5% $NH_3$. [It was therefore not possible to carry out the repeated washing procedure described by Bates *et al.* (1960) and the behaviour of the protein was clearly contrary to that described by these workers.] The precipitate was washed once more with 5% trichloroacetic acid, twice with acetone, twice with ether, and then plated.

### *Isolation of malic dehydrogenase (Table 5, Fig. 3)*

The mitochondria from approx. 30 g. (wet wt.) of liver were incubated in 45 ml. of medium in the presence of radioactive amino acid. The reaction was stopped by cooling the suspension to 0° and adding 1·0 ml. of carrier [$^{12}$C]valine followed by 1·2 ml. of 8% (v/v) Triton X100 in 0·3 M-sucrose. After 10 min. the suspension was centrifuged for 60 min. at 105 000*g*, and the supernatant fluid was loaded directly on a 1·5 cm. × 9·0 cm. calcium phosphate column that had been previously equilibrated with 0·02 M-potassium phosphate, pH 7·0. The column was washed successively with 100 ml. of 0·02 M-phosphate buffer and 40 ml. of 0·08 M-phosphate buffer. Potassium phosphate (0·16 M, pH 7·0) was then run through the column, and after 30 ml. the major peak of malic dehydrogenase appeared. The enzyme was completely eluted by a further 40 ml. of the 0·16 M-phosphate buffer. The peak fractions from the calcium phosphate column were then pooled; 41 ml. was treated with 9·9 g. of ammonium sulphate (40% saturation), and after 20 min. the suspension was centrifuged at 2000*g* for 10 min. The sediment was rejected and a further 12·9 g. of ammonium sulphate added to 46 ml. of the supernatant fluid (80% saturation). After 20 min. at 0° the suspension was centrifuged at 10 000*g* for 30 min. The supernatant was rejected and the sediment resuspended in 7·0 ml. of 0·02 M-phosphate buffer and dialysed against several changes of this buffer for 15 hr. The solution was then loaded on a 1·5 cm. × 9·0 cm. column of the weak cation-exchange resin Zeo-Karb 226 that had been previously equilibrated with 0·02 M-potassium phosphate, pH 7·0. After washing the column the enzyme was eluted with 0·08 M-phosphate buffer, pH 7·0 (Fig. 3).

### *Estimation of malic dehydrogenase*

This enzyme was estimated spectrophotometrically by following the reduction of $DPN^+$ by malate at 340 m$\mu$. One unit was the amount giving an increase in $E_{340}$ of 1·0/min. in a 1·0 cm. light-path cell, containing 2·7 ml. of fluid, the assay being carried out at room temperature. One unit corresponds to the oxidation of 0·43 $\mu$mole of malate/min., assuming a molar extinction coefficient of 6220 for DPNH (Horecker & Kornberg, 1948).

In the experiments on the distribution of malic dehydrogenase in different subcellular fractions (Table 6), the assay method of Beaufay, Bendall, Baudhuin & de Duve (1959*a*) was used, in which the reaction is carried out in the presence of 0·01 M-cyanide. However, some difficulties were experienced with this method because of the formation of a DPN–cyanide complex (cf. Meyerhoff, Ohlmeyer & Mohle, 1938; Colowick, Kaplan & Ciotti, 1951). For all other experiments on malic dehydrogenase, the following reaction conditions were adopted (cf. Thorne, 1960): the reaction mixture consisted of 2·0 ml. of 0·1 M-glycine–NaOH buffer, pH 9·9, 0·3 ml. of 0·18 M-sodium L-malate and 0·3 ml. of DPN (4·5 mg./ml.), and the reaction was started by the addition of 0·1 ml. of enzyme. When the enzyme assayed was in particulate cell fractions (e.g. intact mitochondria or mitochondrial subfractions), the suspension was pretreated with 10 vol. of 0·2% Triton X100 in 0·3 M-sucrose for 30 min. at 0° before assay.

### *Isolation of catalase*

*Large-scale preparation of rat-liver catalase for production of antibody.* The method of Price & Greenfield (1954) for the preparation of rat-liver catalase was followed as far as step 4, fraction P4. Some difficulty was experienced in reproducing the chromatographic purification on calcium phosphate–cellulose columns, but it was found that fraction P4 could readily be purified on columns of diethylaminoethylcellulose (DEAE-cellulose). The partially purified catalase (P4) from 450 g. of liver was dissolved in 22·5 ml. of 0·1 M-$K_2HPO_4$ and the pH adjusted to 4·5 by addition of 3 ml. of 1·0 M-acetate buffer, pH 4·0, followed by M-acetic acid. The precipitate which formed was discarded and the supernatant was dialysed overnight against 2 l. of water. Whatman DEAE-cellulose in a column 2·5 cm. × 15 cm. was equilibrated with 0·025 M-sodium phosphate buffer, pH 7·48, and, after addition of dialysed catalase, the column was developed first with 0·025 M-phosphate buffer, and then with 0·025 M-phosphate buffer containing successively 0·05, 0·1 and 0·2 M-NaCl. Four clearly separated protein peaks were obtained. The second peak was golden brown and had a Katalasefähigkeit (Kat. f.) of 45 000 at 0° when assayed by the method of Sumner & Dounce (1955). This material (200 mg. of protein) was considered to be sufficiently pure to use as an antigen in the production of a specific anti-catalase antibody.

*Preparation of specific precipitating antibody to rat-liver catalase.* An emulsion of 13 mg. of catalase in 2 ml. of Freund's adjuvant (Freund & McDermott, 1942) was injected intramuscularly into an adult rabbit. A week later a second similar injection was given. Four weeks later this was followed by alum-precipitated catalase (Kabat & Mayer, 1948) given intravenously on alternate days in increasing amounts (two doses of 1·25 mg., two of 2·5 mg. and two of 5·0 mg.). Twelve days after the last injection blood

was collected. The serum precipitated catalase quantitatively, 1 ml. of serum being sufficient to precipitate 1·33 mg. of purified catalase.

*Isolation of catalase from mitochondrial fraction by use of specific precipitating antibody.* Mitochondria from 6 g. of liver were incubated with radioactive amino acid. After incubation the mitochondria were centrifuged down at 10 000*g* for 10 min. and the pellet was resuspended in 2·75 ml. of 0·9% NaCl. Butan-1-ol (0·5 ml.) was added slowly at 0° over 30 min., with constant stirring. After a further 30 min. at 0° the suspension was centrifuged at 12 000*g* for 15 min. The supernatant was decanted and extracted three times with cold *n*-hexane and three times with cold ether. The aqueous phase was frozen once to coagulate any denatured protein and the dissolved residual ether removed in a stream of nitrogen. After centrifuging again at 12 000*g* for 15 min., 250 $\mu$g. of ovalbumin was added to the solution, together with sufficient anti-ovalbumin to precipitate this completely. The solution was incubated at 31° for 30 min. and the flocculent precipitate removed by centrifuging. This was done to remove any non-specific material that might become occluded with the catalase–anti-catalase precipitate (Askonas, Campbell, Humphrey & Work, 1954). The amount of catalase in the supernatant was estimated by the method of Sumner & Dounce (1955), and sufficient anti-catalase was added to precipitate it completely. This required 1·34 mg. of antibody/mg. of catalase. After incubation of the mixture for 30 min. at 31°, the specific precipitate was collected, washed twice with saline, resuspended in 5% (w/v) trichloroacetic acid and plated for radioactive assay.

*Protein determinations.* Protein was estimated by its nitrogen content, a micro-Kjeldahl procedure being used. For determining protein content in the effluent from columns, the extinction at 280 m$\mu$ was taken (Warburg & Christian, 1941). In a crude protein mixture eluted from a calcium phosphate column an $E_{280}$ of 1·0 corresponded to 0·95 mg. of protein/ml. as determined by the Kjeldahl procedure; a conversion factor of 0·95 was therefore used to calculate protein concentration (mg./ml.) from extinction measurements.

*Radioactive determinations.* In all experiments, apart from those described for the isolation of cytochrome *c* in Table 2, a zero-time assay was carried out with the mitochondria by adding trichloroacetic acid immediately after the radioactive amino acid. This value for total mitochondrial protein was then subtracted from all the values found for the various proteins studied. Apart from the experiments described in Table 2, all proteins were isolated for plating by the method of Simkin & Work (1957).

When sufficient protein was available it was counted at infinite thickness as described previously (Roodyn *et al.* 1961*a*). When there was not sufficient protein for this, it was dissolved in 0·3–0·4 ml. of 98% formic acid and plated on 2 cm.$^2$ aluminium planchets that had been previously coated with a thin layer of silicone adhesive as described by Martin, Malec, Sved & Work (1961). The planchets were dried overnight over KOH and the amount of protein on the planchet determined by weighing. Counts were corrected to infinite thinness from a calibration curve prepared by plating a standard preparation of total mitochondrial protein of known specific radioactivity. The absolute radioactivity of this standard was determined by counting at infinite thickness in parallel with a standard polymethacrylate (Perspex) source, as described previously (Roodyn *et al.* 1961*a*). With the Geiger–Müller end-window counter used, 1 count/min. at infinite thinness corresponded to 4·4 $\mu\mu$c.

*Materials.* Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Triton X100 was supplied by Charles Lennig and Co., Bedford Row, London, W.C. 1. The cation-exchange resin Zeo-Karb 226 (SRC 44) was a sample kindly supplied by Dr J. A. Hunt; it was described by the manufacturers (The Permutit Co., London, W. 4) as having 2½% of divinylbenzene, and was of approximately 200 mesh. CG-50 was the chromatographic grade of the cation-exchange resin IRC-50 and was supplied by British Drug Houses Ltd., Poole, Dorset. Electrophoretically-pure horse-heart cytochrome *c* was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

## RESULTS

### *Proteins released into medium during incubation*

Between 10 and 20% of the mitochondrial protein escaped into the medium during incubation for 90–120 min. at 30°. The proteins in the medium were not sedimented after 30 min. at 105 000*g*, and were found to be very poorly labelled, having only one-fifth to one-tenth of the specific radioactivity of the total mitochondrial protein. As a result they made a very small contribution to the total radioactivity in the system (Fig. 1). It is unlikely that the release of protein was due to a specific secretory action of the mitochondria, since it occurred equally well in the presence of 0·2 mM-

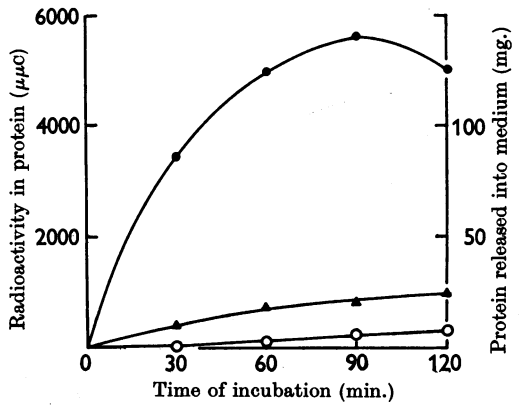


Fig. 1. Rate of labelling of soluble fractions released into medium during incubation. Results are expressed per 100 mg. of total mitochondrial protein. Mitochondria were incubated in presence of 0·33 $\mu$c of $^{14}$C-labelled algal-protein hydrolysate/ml. and sedimented at 10 000*g* for 10 min. at the times shown, and the radioactivity in the protein of the supernatant and sediment was determined. ▲, Protein released; ○, radioactivity of protein in medium; ●, radioactivity of protein in mitochondria.

Table 1. *Chromatography on calcium phosphate of proteins released into medium during incubation*

Mitochondria were incubated for 90 min. in the presence of 0·33 $\mu$c of $^{14}$C-labelled algal-protein hydrolysate/ml. After incubation the mitochondria were sedimented at 10 000*g* for 10 min. and the supernatant was loaded on a 1 cm. × 14 cm. calcium phosphate column. The proteins were eluted with increasing concentrations of sodium phosphate buffer, pH 7·0. Penicillin and streptomycin were present in the incubation medium.

| Fraction | Phosphate concn. in eluate (M) | Protein (mg.) | Radioactivity in protein ($\mu\mu$c/mg.) | Radioactivity in protein (total $\mu\mu$c) |
|---|---|---|---|---|
| Total mitochondrial proteins | — | 204·8 | 131·5 | 26 958 |
| Proteins released into medium | — | 19·8 | 43·3 | 859 |
| Proteins eluted from column | 0·04 | 0·5 | 22·5 | 11 |
| | 0·10 | 4·0 | 13·2 | 53 |
| | 0·17 | 3·3 | 19·0 | 63 |
| | 0·24 | 4·0 | 19·8 | 79 |
| | 0·34 | 4·7 | 27·0 | 127 |
| | 0·41 | 1·7* | 34·1 | 58 |
| | 0·70 | 0·1 | 19·4 | 2 |

* Probably rich in cytochrome *c*, see text.

2,4-dinitrophenol, when oxidizable substrate was omitted from the medium, or simply when the mitochondria were incubated in the sucrose–EDTA–nicotinamide isolation medium.

This soluble-protein fraction was pale yellow (cf. Hogeboom & Schneider, 1950), and, when it was loaded on a calcium phosphate column, a reddish-pink band appeared at the origin. The column was developed with stepwise increases in phosphate buffer concentration, and none of the eluted proteins had a specific radioactivity as high as that of the total mitochondrial protein (Table 1). Although the bulk of the protein loaded on the column was eluted, only about half of the radioactivity was recovered, suggesting that some highly radioactive material had been retained (D. B. Roodyn, in preparation). Elution with 0·41 M-phosphate buffer removed the reddish-pink band from the column, and the effluent had a clear absorption peak at 415 m$\mu$ and a faint peak at 550 m$\mu$ after reduction with sodium dithionite. When the protein solution was concentrated by dialysis against polyethylene glycol (Kohn, 1959), a reddish-brown precipitate appeared. It was very likely, therefore, that this fraction was rich in cytochrome *c*. However, it had a specific radioactivity less than a third of that of the mitochondria and contributed to only 0·2 % of the total radioactivity. Since this result was clearly at variance with the results of Bates *et al.* (1958, 1960), the radioactivity of more highly purified cytochrome *c* was examined.

*Cytochrome* c. Three experiments were carried out (Table 2). In the first, the mitochondria were centrifuged down after incubation in the presence of radioactive amino acid and cytochrome *c* was isolated from the pellet only (see Methods section). The cytochrome *c* was plated directly, without further washing, and contained only 0·7 % of the total radioactive protein in the system. Very nearly the same amount was found in the control in which oxidizable substrate was omitted, so that the energy-dependent incorporation into cytochrome *c* was only 1·6 $\mu\mu$c out of a total of energy-dependent incorporation of 2100 $\mu\mu$c.

Since this negative result could have been due to the loss of cytochrome *c* into the medium, the procedure was modified so that both medium and mitochondria were extracted. The radioactivity of the protein at the various stages in the purification was followed (Table 2, Expt. 2) and the specific radioactivity progressively fell as the cytochrome was purified, until finally the cytochrome *c* eluted from the ion-exchange resin had a specific radioactivity only 11·5 % of that of the total mitochondrial proteins, and contained only 8·7 $\mu\mu$c of a total radioactivity of 40 200 $\mu\mu$c. It was thought possible that even this small amount of radioactivity was due to non-specific adsorption of radioactive amino acid. In the third experiment, therefore, the protein was treated more thoroughly with carrier and trichloroacetic acid before plating (see Methods section), and its specific radioactivity then fell to 3·1 % of the total protein. In addition, in a control experiment (Table 2, Expt. 3*b*) in which mitochondria were kept at 0° for 90 min. and then mixed with radioactive amino acid, the cytochrome *c* also had some residual radioactivity, so that the 'true' incorporation was only 0·9 (i.e. 2·7–1·8) $\mu\mu$c out of a total true incorporation in the mitochondria of 26 400 $\mu\mu$c. The 'true' specific radioactivity (1·7 $\mu\mu$c/mg.) was only one-hundredth that of the mitochondria (182 $\mu\mu$c/mg.).

Because of the small quantity of material available, it was not possible to make an accurate estimate of the purity of the isolated cytochrome *c*. The protein in Expt. 2 (Table 2) eluted from the ion-exchange column showed absorption peaks at

415, 520 and 550 mμ after reduction with sodium dithionite. The proportions of the extinctions at these three wavelengths (100:11·8:19·0) were similar to those determined simultaneously with horse-heart cytochrome *c* (100:12·8:20·5). The positions of the maxima and the proportions are in moderate agreement with the results given by Margoliash & Frohwirt (1959) for horse-heart cytochrome *c* (maxima for reduced cytochrome *c*: 416, 520·5 and 550·25 mμ; proportions at 415, 520 and 550 mμ 100:12·4:21·6). It was probable, therefore, that the preparation was moderately pure. However, because of the negligible radioactivity present in it, there seemed little point in improving the preparation further.

*Fractionation of detergent-soluble proteins*

The distribution of radioactivity in various mitochondrial subfractions obtained after disruption with the neutral detergent Triton X 100 is given in Table 3. Although nearly half the protein is found in the detergent-soluble proteins (i.e. the super-

Table 2. *Isolation of cytochrome* c *from mitochondria labelled* in vitro

See Methods section for details of the isolation and washing of the cytochrome *c* in each experiment. In Expt. 1, mitochondria were incubated in the presence of 0·2 μc of [$^{14}$C]valine/ml. for 90 min. with succinate present (*a*) or absent (*b*) from the reaction mixture. Penicillin and streptomycin were present. In Expt. 2, mitochondria were incubated in the presence of 0·5 μc of $^{14}$C-labelled algal-protein hydrolysate/ml. for 90 min. Penicillin was present. A zero-time assay for the total mitochondrial protein gave 7·3 μμc/mg. and this was subtracted from all the values. In Expt. 3*a*, mitochondria were incubated in the presence of 0·5 μc of $^{14}$C-labelled algal-protein hydrolysate/ml. for 90 min.; penicillin was present. In Expt. 3*b*, an equal amount of the same suspension was kept at 0° for 90 min., 0·5 μc of $^{14}$C-labelled algal-protein hydrolysate/ml. added and cytochrome *c* immediately extracted with $H_2SO_4$.

| Expt. | Fraction | Protein (mg.) | Radioactivity in protein (μμc/mg.) | Radioactivity in protein (total μμc) |
|---|---|---|---|---|
| 1*a* | Total protein | 125 | 23·5 | 2940 |
| | Residual protein after $H_2SO_4$ extraction | — | 34·0 | — |
| | Cytochrome *c* | — | — | 20·6 |
| 1*b* | Total protein | 125 | 6·7 | 838 |
| | Residual protein after $H_2SO_4$ extraction | — | 9·1 | — |
| | Cytochrome *c* | — | — | 19·0 |
| 1*a* − 1*b* | Total protein | — | — | 2102 |
| | Cytochrome *c* | — | — | 1·6 |
| 2 | Total protein | 244 | 165·0 | 40 200 |
| | Residual protein after $H_2SO_4$ extraction | — | 191·0 | — |
| | Crude protein extracted with $H_2SO_4$ | — | 63·0 | — |
| | Cytochrome *c* | — | 22·1 | — |
| | Cytochrome *c* eluted from IRC-50 resin | 0·46 | 19·0 | 8·7 |
| 3*a* | Total protein | 145 | 193·0 | 28 000 |
| | Residual protein after $H_2SO_4$ extraction | — | 202·0 | — |
| | Cytochrome *c* | 0·45 | 6·1 | 2·7 |
| 3*b* | Total protein | 145 | 11·0 | 1600 |
| | Residual protein after $H_2SO_4$ extraction | — | 11·0 | — |
| | Cytochrome *c* | 0·41 | 4·4 | 1·8 |
| 3*a* − 3*b* | Total protein | — | 182·0 | 26 400 |
| | Cytochrome *c* | — | 1·7 | 0·9 |

Table 3. *Distribution of protein, radioactivity and malic dehydrogenase in mitochondrial sub-fractions*

Results are the means of three experiments and are expressed per 100 mg. of mitochondrial protein. Mitochondria were incubated for 90 min. with 0·2 μc of [$^{14}$C]valine/ml., centrifuged down for 10 min. at 10 000*g* and disrupted with 0·2 % Triton X 100. The disrupted mitochondria were then sub-fractionated by centrifuging at the forces shown. Penicillin and streptomycin were present in the incubation media.

| Fraction | Protein (mg.) | Radioactivity in protein (μμc/mg.) | Radioactivity in protein (total μμc) | Malic dehydrogenase (units/mg.) | Malic dehydrogenase (total units) |
|---|---|---|---|---|---|
| Total mitochondrial protein | 100·0 | 48·8 | 4880 | 4·10 | 410·0 |
| Sediment after 30 min. at 10 000*g* | 17·4 | 108·2 | 1880 | 0·45 | 7·8 |
| Sediment after 60 min. at 105 000*g* | 14·3 | 106·0 | 1520 | 2·30 | 33·0 |
| Supernatant after 60 min. at 105 000*g* | 46·6 | 20·0 | 930 | 5·32 | 249·0 |
| Proteins released into medium during incubation | 17·3 | 11·2 | 190 | 4·35 | 75·2 |
| Recovery | 95·6 | | 4520 | | 365·0 |

Table 4. *Chromatography on calcium phosphate of proteins released by detergent*

The results are taken from the same experiment as that given in Table 1. The sedimented mitochondria were disrupted with 0·2% Triton X 100 and centrifuged at 105 000*g* for 60 min. The supernatant was loaded on a 1 cm. × 14 cm. calcium phosphate column and the proteins eluted with increasing concentrations of sodium phosphate buffer, pH 7·0.

| Fraction | Phosphate concn. in eluate (M) | Protein (mg.) | Radioactivity in protein ($\mu\mu$C/mg.) | Radioactivity in protein (total $\mu\mu$C) | Malic dehydrogenase (total units) |
|---|---|---|---|---|---|
| Total mitochondrial protein | — | 204·8 | 131·5 | 26 958 | 689 |
| Proteins released by detergent | — | 87·0 | 29·5 | 2560 | 340 |
| Proteins eluted from column | 0·04 | 9·6 | 20·8 | 200 | 0 |
| | 0·10 | 28·0 | 14·6 | 410 | 370 |
| | 0·15 | 21·1 | 16·8 | 355 | 30 |
| | 0·22 | 10·8 | 42·1 | 456 | 5 |
| | 0·34 | 5·2 | 76·0 | 394 | 0 |
| | 0·40 | 3·0 | 86·8 | 260 | 0 |
| | 0·71 | 4·1 | 162·0 | 660 | 0 |

natant after 60 min. at 105 000*g*), these contain only about 20% of the total radioactivity. The bulk of the radioactivity is found in the material insoluble in detergent.

Fractionation by batchwise elution of the detergent-soluble proteins (Table 4) showed that the radioactivity was spread rather diffusely through the effluent fractions, with a small amount of protein of relatively high specific radioactivity being eluted with high phosphate concentrations (0·71 M). Malic dehydrogenase, the bulk of which is in the detergent-soluble fraction (Table 3), appeared very sharply in the fraction eluted with 0·1 M-phosphate buffer. This fraction contained over 50% of the total mitochondrial enzyme, but only 1·5% of the total radioactivity. The experiment was repeated with gradient-elution chromatography and a longer column (Fig. 2). Although a series of protein peaks was obtained, the radioactivity was spread irregularly throughout the chromatogram. As in Table 4, the protein eluted at the end of the chromatogram with the highest concentration of buffer had a relatively high radioactivity. However, the bulk of the proteins were poorly labelled, since fractions 1–18 contained 16·4% of the total mitochondrial protein, but only 4·4% of the total radioactivity, so that the average specific radioactivity was only 0·27 that of the mitochondria. Malic dehydrogenase appeared on the chromatogram as a sharp peak, but there was no evidence of a radioactive peak in the same region in the chromatogram. Fractions 8–10 contained 53% of the total mitochondrial enzyme but only 0·9% of the radioactivity.

*Malic dehydrogenase.* As with cytochrome *c*, these indications of poor rates of labelling were fully confirmed by isolation of a more purified protein. The radioactivities of the proteins at each step in the purification are given in Table 5 and the final purification on the cation-exchange resin Zeo-Karb 226 is shown in Fig. 3. As the enzyme was purified, the radioactivity progressively fell until the purest fraction (fraction 12), which contained 31% of the total enzyme and a specific enzyme activity 100 times that of the mitochondria, had no significant radioactivity.

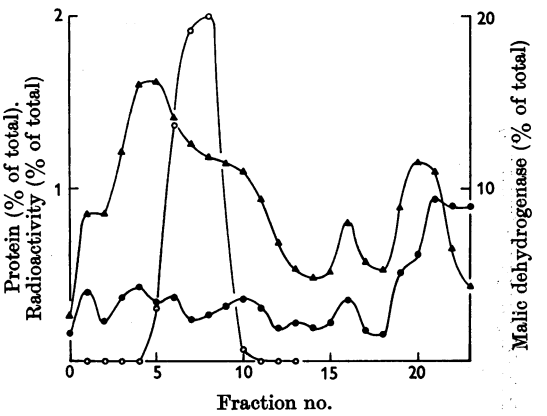


Fig. 2. Chromatography on calcium phosphate of proteins released by detergent. Results are expressed as percentage of the total amount in the mitochondria. Mitochondria containing 106 mg. of protein were incubated for 90 min. with 0·2 $\mu$C of [$^{14}$C]valine/ml. and then disrupted with 0·2% Triton X 100. The suspension was centrifuged at 105 000*g* for 60 min. and the supernatant, containing 41·6 mg. of protein, was loaded on a 1·0 cm. × 26·5 cm. calcium phosphate column. The column was washed with 0·02 M-sodium phosphate, pH 7·0, and the proteins were eluted with a gradient from 0·02 to 0·2 M-phosphate buffer (fractions 1–15) followed by a gradient from 0·2 to 0·5 M-phosphate buffer (fractions 15–23); 10 ml. fractions were collected. The original mitochondrial fraction had a specific radioactivity of 41 $\mu\mu$C/mg. and 3·72 units of malic dehydrogenase/mg. of protein. Penicillin and streptomycin were present in the incubation medium. ▲, Protein; ●, radioactivity in protein; ○, malic dehydrogenase.

Table 5. *Isolation of malic dehydrogenase from mitochondria labelled* in vitro

Mitochondria were incubated for 2 hr. in the presence of 0·2 $\mu$c of [$^{14}$C]valine/ml., and malic dehydrogenase was isolated (see Methods section).

| Fraction | Protein (mg.) | Radioactivity in protein ($\mu\mu$c/mg.) | Radioactivity in protein (total $\mu\mu$c) | Malic dehydrogenase (units/mg.) | Malic dehydrogenase (total units) |
|---|---|---|---|---|---|
| Total mitochondrial protein | 344 | 93·1 | 32 100 | 3·03 | 1040 |
| 105 000*g* supernatant after disruption with Triton X 100 | 205 | 15·1 | 3100 | 5·70 | 1160 |
| Pooled eluate from calcium phosphate column | — | 1·4 | — | — | 840 |
| Ammonium sulphate fractionation (40–80% saturation) | 43 | 0·7 | 30 | 12·3 | 530 |
| Chromatography on Zeo-Karb 226* | | | | | |
| 0·02M-phosphate (fractions 2, 3) | 12·7 | 0·8 | 10 | 0·6 | 8 |
| 0·08M-phosphate (fraction 12) | 1·05 | 0·0 | 0 | 309·0 | 325 |
| 0·08M-phosphate (fraction 13) | 1·05 | 0·0 | 0 | 155·0 | 163 |

* See Fig. 3.

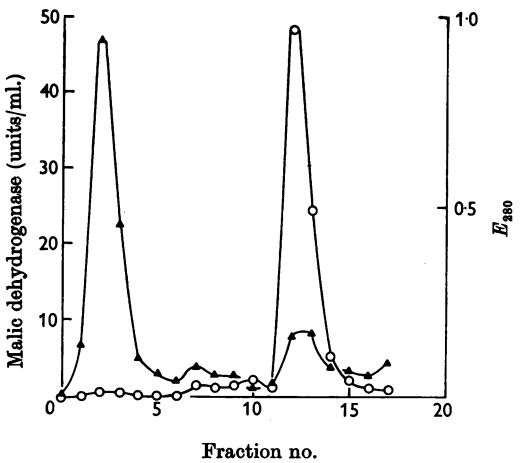


Fig. 3. Chromatography of malic dehydrogenase on the weak cation-exchange resin Zeo-Karb 226. Crude enzyme (32·2 mg.) was loaded on a 1·5 cm. × 9·0 cm. column (fractions 0 and 1). The column was washed with increasing concentrations of potassium phosphate buffer, pH 7·0: 0·02M in fractions 2–5; 0·04M in fractions 6–9; and 0·08M in fractions 10–17. Fractions 0–9 were 10 ml., and fractions 10–17 were 5 ml. each in volume. ▲, $E_{280}$; ○, malic dehydrogenase.

Shortage of material prevented an accurate assessment of the degree of purity of the enzyme at the final stage, but, from the turnover numbers given by Wolfe & Neilands (1956) and Grimm & Doherty (1961) for purified malic dehydrogenase under similar assay conditions, it was calculated that the enzyme was probably 30–40% pure. Since it contained no detectable radioactivity, however, there was little point in purifying it further.

Some experiments were carried out to examine the intracellular localization of malic dehydrogenase. Fractionation of liver homogenates showed that about 40% of the enzyme was in the mitochondrial fraction and about 40% in the cell sap (Table 6). Repeated washing of the mitochondrial fraction released only a small amount of enzyme, and, when the final suspension of washed mitochondria was centrifuged at 700*g* for 6 min. to remove any residual nuclei or red blood cells, a very small loss in enzyme actually occurred. Very little enzyme was found in the microsomal fraction and only about 10% of the total in the crude nuclear plus whole-cell fraction. It was unlikely, therefore, that the enzyme in the mitochondrial fraction was due to the presence of whole cells, nuclei, red blood cells or microsomes; nor was it due to contamination by enzyme from the soluble fraction because of insufficient washing. The absence of soluble malic dehydrogenase, as distinct from the mitochondrial enzyme, is confirmed by the complete retention of the enzyme on a weak cation-exchange resin as shown in Fig. 3 (Thorne, 1960).

*Catalase.* As with cytochrome *c* and malic dehydrogenase, the catalase isolated from mitochondria labelled *in vitro* had a negligible radioactivity, the specific radioactivity after 1 hr. of incubation being only 0·4% of that of the total mitochondrial protein (Table 7).

As a check that the immunological method of isolation used would give radioactive catalase, were this formed, a 350 g. rat was given an injection of 8 $\mu$c of $^{14}$C-labelled algal-protein hydrolysate and killed 2½ hr. later. An homogenate was made from 10 g. of liver and the mitochondria were isolated in the usual way. Catalase isolated by precipitation with anti-catalase after clearing with

Table 6. *Distribution of malic dehydrogenase in subcellular fractions from rat-liver homogenate*

The results are the mean of two experiments and are expressed per 100 mg. (wet wt.) of liver mince or for the amount of fraction derived from this. Mitochondria were washed by repeated centrifuging at 10 000*g* for 10 min. Malic dehydrogenase was estimated by the method of Beaufay *et al.* (1959*a*).

| Fraction | Malic dehydrogenase (units/100 mg. of liver) | Malic dehydrogenase (% of total) |
|---|---|---|
| (1) Distribution in major cell fractions | | |
| Total homogenate | 6·58 | 100·0 |
| Nuclei + whole cells + red blood cells | 0·69 | 10·5 |
| Unwashed mitochondrial fraction | 3·00 | 45·6 |
| Microsomes + cell sap | 2·56 | 39·2 |
| Microsomes | 0·18 | 2·7 |
| Cell sap | 2·29 | 34·6 |
| (2) Purification of mitochondrial fraction | | |
| Supernatant after 1st wash | 0·21 | 3·2 |
| Supernatant after 2nd wash | 0·07 | 1·0 |
| Supernatant after 3rd wash | 0·06 | 0·9 |
| Supernatant after 4th wash | 0·03 | 0·5 |
| Sediment after centrifuging washed mitochondria at 700*g* for 6 min. | 0·09 | 1·4 |
| Washed mitochondrial fraction | 2·32 | 35·3 |

Table 7. *Isolation of catalase from mitochondria labelled* in vivo *and* in vitro

Labelling *in vivo* was carried out by injecting 8 $\mu$c of $^{14}$C-labelled algal-protein hydrolysate into a 350 g. rat. Catalase was isolated from the mitochondria in 10 g. of liver. Labelling *in vitro* was carried out by incubating mitochondria from 6 g. of liver with 2 $\mu$c of $^{14}$C-labelled algal-protein hydrolysate.

| Condition of labelling | Radioactivity in protein ($\mu\mu$c/mg.) Total mitochondrial protein | Catalase |
|---|---|---|
| *In vivo* (2·5 hr.) | 765 | 600 |
| *In vitro* (15 min.) | 109 | 2·3 |
| (30 min.) | 179 | 1·9 |
| (60 min.) | 183 | 0·7 |

ovalbumin–anti-ovalbumin, as in the experiment *in vitro*, was found to be highly radioactive, having approximately the same specific radioactivity as the total mitochondrial protein (Table 7).

## DISCUSSION

It is clear from the above results that the observed amino acid incorporation by rat-liver mitochondria *in vitro* is not due to the synthesis of a small number of highly radioactive soluble proteins. The bulk of the radioactivity is incorporated into the insoluble proteins [Table 3; see also Roodyn (1961); Roodyn *et al.* (1961*a*)]. In chromatographic analysis on calcium phosphate, the small amount of radioactivity in the soluble proteins was diffusely spread throughout the chromatograms and there was no evidence of sharp radioactive peaks, although clear separation of the various proteins undoubtedly occurred (cf. the malic-dehydrogenase peak in Fig. 2). The only fractions of significant radioactivity were obtained by elution with high concentrations of phosphate buffer (Table 4, fraction eluted with 0·7 M-phosphate buffer; Fig. 2, fractions 19–24). Subsequent work has shown that this is due to the slow release of small amounts of a relatively insoluble protein of high specific radioactivity that is tightly bound to calcium phosphate (D. B. Roodyn, in preparation). The bulk of the freely soluble mitochondrial proteins are eluted well before this material, however, and contain very little radioactivity.

It is well established that the soluble-protein fraction of liver mitochondria contains many components (Schneider, 1959). Hogeboom & Schneider (1950) demonstrated three or four components by ultracentrifugal analysis, and at least five different proteins have been shown by electrophoresis (de Lamirande, Allard & Cantero, 1953; Caraviglios & Franzini, 1959). Examples of proteins detected in this fraction are: adenylate kinase (Kielley & Kielley, 1951), catalase (Feinstein, 1959), cytochrome *c* (Hogeboom & Schneider, 1952), deoxyribonuclease (Schneider & Hogeboom, 1952), DPNH–cytochrome *c* reductase (Hogeboom & Schneider, 1950), DPN- and TPN-linked isocitric dehydrogenases (McMurray, Maley & Lardy, 1958), fumarase (Kuff, 1954), glutamic dehydrogenase (Hogeboom & Schneider, 1953; McMurray

*et al.* 1958; Bendall & de Duve, 1960), malic dehydrogenase (Bendall & de Duve, 1960), ribonuclease (Schneider & Hogeboom, 1952) and TPNH–cytochrome *c* reductase (Reynafarje & Potter, 1957). Because of the small amount of radioactivity found in the soluble-protein fraction, it can be inferred that the observed amino acid incorporation *in vitro* was not due to the exclusive synthesis of any one of the above proteins.

The negligible rates of labelling of catalase, malic dehydrogenase and cytochrome *c* were observed under optimum conditions for the incorporation of radioactive amino acids into mitochondria (Roodyn *et al.* 1961*a*). This could be explained if these enzymes were not truly mitochondrial. Their intracellular localization is therefore pertinent to the problem.

The results with catalase are the most equivocal of the three enzymes studied, since the amount of enzyme found in the mitochondrial fraction appears to vary greatly with the method of preparation. Euler & Heller (1949) found only 16–23% of the total enzyme in the mitochondrial fraction. Ludewig & Chanutin (1950), however, found about 45% and Greenfield & Price (1956) reported that 63% of the catalase could be recovered in the mitochondrial fraction, provided that polyvinylpyrrolidone was added to prevent the escape of enzyme into the cell sap. The ease with which catalase from the mitochondrial fraction becomes redistributed in other fractions was confirmed by Adams & Burgess (1959). There is also some doubt as to whether the catalase present in the mitochondrial fraction is associated with mitochondria. Thomson & Klipfel (1957) observed that catalase and uricase sediment closely together during gradient-density centrifuging in a particle fraction lighter than the particles containing succinic dehydrogenase and cytochrome oxidase, enzyme 'markers' for mitochondria. de Duve *et al.* (1960) have also observed a close relationship in the sedimentation properties of particles containing uricase, catalase and D-amino acid oxidase, and suggest that these enzymes are present in subcellular particles specially involved in hydrogen peroxide metabolism and distinct from both mitochondria and lysosomes. It is therefore possible that the reason for the failure to obtain synthesis of catalase is that these particles differ from mitochondria in their requirements for protein synthesis.

The relationship between malic dehydrogenase and mitochondria is better established, however. Beaufay *et al.* (1959*a*) found 60% of the total enzyme in the heavy mitochondrial fraction, 11% in the light, very little in the microsomal fraction and 11% in the cell sap. A similar distribution has been observed in the above work (Table 6) except that the enzyme is more evenly distributed between mitochondria and cell sap. Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959*b*) showed that particles containing malic dehydrogenase had sedimentation properties in gradient-density centrifuging identical with those of particles containing cytochrome oxidase, glutamic dehydrogenase and alkaline deoxyribonuclease. These particles contributed 80–90% of the protein in the mitochondrial fraction and undoubtedly were true mitochondria, rather than lysosomes or catalase-rich particles. It is not certain whether the enzyme present in the cell sap is derived from the mitochondria during fractionation or is a different protein entirely. The mitochondrial and soluble enzymes have been shown to be different in many respects (kinetic properties, inhibition by high concentrations of oxaloacetate, behaviour on electrophoresis and chromatography, and in immunological characteristics) by a number of workers (e.g. Delbruck, Zebe & Bucher, 1959; Thorne, 1960; Siegel & Englard, 1960; Grimm & Doherty, 1961). However, Sophianopoulos & Vestling (1960) have shown that treatment of the soluble enzyme with butan-1-ol changes its chromatographic behaviour to that of the mitochondrial enzyme, and that after chromatography the soluble enzyme then has the same behaviour on electrophoresis as the mitochondrial enzyme. Until the relationship between these two enzymes is clarified, therefore, the possibility remains that the reason for the failure to observe synthesis of the mitochondrial enzyme is that the soluble enzyme is made first elsewhere in the cell, and then transferred to the mitochondria.

Of the three proteins studied, the relationship between mitochondria and cytochrome *c* is the best established. The bulk of the cytochrome *c* is found in the mitochondrial fraction (Schneider & Hogeboom, 1950; Dianzani & Viti, 1955) and the amount found in other fractions is most probably due to contamination (Beinert, 1951). The role of cytochrome *c* in mitochondrial oxidations has been established by a large number of workers and there is no doubt that it plays an essential part in the process of oxidative phosphorylation, the characteristic biochemical property of the mitochondrion (Slater, 1958).

The question whether cytochrome *c* is synthesized by the mitochondrion is therefore important. The results obtained above are in complete disagreement with those of Bates *et al.* (1958, 1960). These authors reported that, under conditions similar to those used in this work, after incubation of rat-liver mitochondria for only 20 min., the cytochrome *c* had a radioactivity of up to 875 counts/min./mg. and that this value was 20–40 times higher than the total mitochondrial protein after extraction of the cytochrome. There is some

confusion as to the yield of cytochrome *c* obtained by Bates *et al.* (1958, 1960). In the 1958 publication a yield of 0·15 μmole (or 1·95 mg., assuming a mol.wt. of 13 000) is given for 24 g. of liver. In the 1960 publication, in a very similar experiment the yield was given as only 0·017–0·023 μmole (0·22–0·30 mg.), also from 24 g. of liver (containing 90 mg. of mitochondrial protein). If the first value is correct, it can be calculated that about 40% of the total radioactivity in the system appeared in cytochrome *c*. Even if the lower figure is correct, between 4 and 12% of the total radioactivity appears in cytochrome *c*. In our three consecutive experiments, in which the washing procedure of the cytochrome was steadily improved, we observed 0·076, 0·022 and 0·0034% of the total radioactivity in the cytochrome *c*. We have never observed specific radioactivities of the order reported by Bates *et al.* even in the crudest preparations. For example, the crude protein fraction extracted by the initial acid treatment had only 14·3 counts/min./mg. (Table 2). The maximum radioactivity found in cytochrome *c* was 4·3 counts/min./mg. In Expt. 3 (Table 2) it was only 1·4 and, after correction for the zero-time sample, fell to the negligible value of 0·4 count/min./mg.

The differences in our methods and those of Bates *et al.* (1958, 1960) are probably insufficient to explain the different results. For example, we have omitted cell sap from our system, replacing it with a mixture of amino acids, AMP and DPN (Reis *et al.* 1959*a*; Roodyn *et al.* 1961*a*), whereas Bates *et al.* used cell sap for the experiments with rat-liver mitochondria. However, in experiments with calf-heart sarcosomes (Bates & Simpson, 1959), very high radioactivities are reported in cytochrome *c* after incubation in the absence of cell sap in a system very similar to that used in this paper. Bates *et al.* (1960) mention that cell sap is not inactivated by boiling and that its action is most probably due to its RNA and amino acid content. Nor can the differences in results be ascribed to the methods of isolation of cytochrome *c*, since in both cases they were based on the extraction procedure of Rosenthal & Drabkin (1943), the precipitation procedure of Keilin & Hartree (1937) and the final chromatographic purification of Palêus & Neilands (1950). Because of the small yield of material from the incubated mitochondria, the isolation procedure had been somewhat simplified in our case, and to reduce adsorptive losses a small column of ion-exchange resin IRC-50 was used (1 cm. × 1·5 cm., instead of 0·9 cm. × 16 cm. used by Bates *et al.* 1960). From the spectroscopic and chromatographic data presented by these authors, it would seem that the cytochrome *c* that they isolated was purer than ours. The very high radioactivities they reported cannot therefore be reasonably ascribed to the presence of contaminant material since our (less pure) enzyme had virtually no radioactivity. Since this work was completed we have been informed by Dr M. V. Simpson that he has obtained results essentially in agreement with ours (Simpson, Skinner & Lucas, 1961).

The failure to demonstrate the synthesis *in vitro* of soluble mitochondrial proteins could mean either that the conditions used, although optimum for amino acid incorporation, are not suitable for synthesis of these proteins, or that these proteins are not normally made by the mitochondrion but are transferred to it during the process of mitochondrial replication. The general aspects of the relationship between protein synthesis and mitochondrial replication have been discussed elsewhere (Roodyn *et al.* 1961*b*), and, until more is known of the site of synthesis of mitochondrial proteins in the living cell, the significance of the negligible rates *in vitro* for soluble proteins reported above cannot be accurately assessed. Work is in progress to characterize the proteins that are synthesized by isolated mitochondria, and evidence has been obtained (D. B. Roodyn, in preparation) that the major part of the incorporation is into a relatively insoluble lipoprotein, possibly related to the structural protein reported by Green, Tisdale, Criddle, Chen & Bock (1961).

## SUMMARY

1. Isolated rat-liver mitochondria were incubated in the presence of radioactive amino acids and the rate of incorporation into the soluble proteins was examined.

2. The soluble proteins, that had either escaped into the medium during incubation, or that were released by the disruption of the mitochondria with a neutral detergent, were poorly labelled compared with the insoluble mitochondrial proteins.

3. Fractionation of the crude soluble-protein fraction by chromatography on calcium phosphate showed that the small amount of radioactivity present was diffusely spread throughout the fractions, no single protein being of very high specific radioactivity.

4. An immunological method for the isolation of catalase from rat-liver mitochondria was developed and it was shown that the incorporation *in vitro* into this protein was negligible.

5. A method for the 100-fold purification of malic dehydrogenase is also described, and it was shown that this enzyme was not labelled to a significant extent.

6. Cytochrome *c* isolated from mitochondria labelled *in vitro* was also found to have a negligible radioactivity.

7. It was concluded that the synthesis of specific soluble mitochondrial proteins is not carried out by isolated mitochondria.

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