SUMMARY

1. Mannoheptulose has been shown to decrease the net uptake of glucose and of mannose by rat-liver slices.

2. This effect has been attributed to an inhibition of hexose phosphorylation in the liver because inhibition of glucokinase and hexokinase by mannoheptulose in extracts of rat liver has been demonstrated.

3. The possibility that a similar inhibition of glucose phosphorylation in β-cells of isolated rabbit pancreas could contribute to inhibitory effects of mannoheptulose on glucose-induced insulin secretion, observed previously, is discussed.

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Biochem. J. (1964) **91**, 59

The Fractionation of Proteins from Ox-Heart Mitochondria Labelled *in vitro* with Radioactive Amino Acids

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The incorporation of radioactive amino acids into protein by isolated ox-heart mitochondria has been described by Simpson, Skinner & Lucas (1961) and Truman & Löw (1963), incorporation by submitochondrial particles from ox heart has been demonstrated by Kalf & Simpson (1959) and Kroon (1963), and mitochondria from various other sources have been found capable of incorporating amino acids into their proteins (McLean, Cohn, Brandt & Simpson, 1958; Roodyn, Reis & Work, 1961*a*; Truman & Korner, 1962; Kalf, 1963), but there is still little evidence about which of the mitochondrial proteins becomes labelled and of whether the incorporation represents the synthesis of any specific protein. Roodyn, Suttie & Work (1962) have studied the proteins labelled by the incorporation of radioactive amino acids into isolated rat-liver mitochondria, which they fractionated to yield purified catalase, malate dehydrogenase and cytochrome *c*. However, they found no significant incorporation into any of these proteins, and Roodyn (1962) concluded that the major site of the incorporation of amino acids was into insoluble lipoprotein. Simpson *et al.* (1961) were unable to demonstrate any incorporation of radioactive valine into cytochrome *c* in isolated ox-heart mitochondria.

In the experiments described below the proteins of ox-heart mitochondria were fractionated after incubation of the mitochondria with ^{14}C-labelled amino acids. Ox-heart mitochondria were chosen because they were available in sufficient quantities and showed an appreciable incorporation of amino acid into protein under relatively simple incubation

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conditions. The methods of fractionation used included differential centrifugation, fractional precipitation with ethanol, ion-exchange chromatography and density-equilibrium centrifugation. The results are consistent with the view that a relatively non-polar soluble protein is the principal site of the incorporation of amino acids in isolated mitochondria.

MATERIALS AND METHODS

Radioactive amino acids. DL-[1-^{14}C]Leucine (specific activity 1·5 mC/m-mole) was obtained from the New England Nuclear Co., Boston, Mass., U.S.A. Generally ^{14}C-labelled algal-protein hydrolysate (specific activity 0·2 mC/mg.) was from The Radiochemical Centre, Amersham, Bucks., and was purified by adsorption on to a column of Dowex 50 ion-exchange resin previously equilibrated with 0·1 N-HCl, followed by elution with 4 N-HCl.

Mitochondria. Ox hearts from the slaughterhouse were used and the mitochondria were prepared by the method of Löw & Vallin (1963).

Incubation conditions. The mitochondria were labelled by incubation with 0·5 μC of radioactive amino acid/ml., in the presence of tris–HCl buffer, pH 7·4 (35 mM), KCl (80 mM), $MgCl_2$ (10 mM) and sucrose (125 mM), as described by Truman & Löw (1963). At the end of the incubation the mitochondrial suspension was cooled to 0° and centrifuged at 10 000g for 15 min., and the pellet so obtained was used for the subsequent fractionations.

Disruption of the mitochondria. The mitochondrial pellet after incubation was suspended in a medium appropriate to the fractionation to be carried out (see below) and was then subjected to ultrasonic vibrations. When the fractionation was by differential centrifugation the mitochondria were frozen and thawed once before ultrasonic treatment as this had been found to improve the yield without affecting the respiratory activity of the particles obtained (Löw & Vallin, 1963), but it was later found that freezing caused some denaturation of the proteins and so it was avoided in all fractionations other than that by differential centrifugation.

Ultrasonic treatment was carried out at a protein concentration of 40 mg./ml., and when relatively large volumes were treated (more than 10 ml.), before fractionation by differential centrifugation, ethanol precipitation or column chromatography, a Raytheon sonicator was used at 10 kcyc./sec. When smaller volumes were used, in the density-equilibrium centrifugation method, ultrasonic treatment was carried out in an MSE sonicator working at 16 kcyc./sec. and 1·4 A.

Protein determinations. Protein concentrations of solutions were determined by the biuret method of Gornall, Bardawill & David (1949), except for the fractions from density-gradient experiments when the extinctions at 260 mμ and 280 mμ were determined after the addition of sodium deoxycholate (final concn. 1%) to the fractions to render them clear. The protein concentration was calculated from the extinction coefficients by using the nomogram of E. Adams (distributed by the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.). The results of such determinations were checked by the biuret method on some fractions, and agreement between the two methods was within 10%.

The preparation of protein samples and measurement of their radioactivity were as described by Truman & Korner (1962), and specific activities were calculated from the weight of the isolated protein, with corrections for self-absorption and background counts. Sufficient counts were recorded to ensure a standard error of less than 3%, and 1 count represented about 2·5 $\mu\mu$C.

Fractionation by differential centrifugation. The method was that used by Löw & Vallin (1963) to prepare phosphorylating electron-transport particles. The pellet of mitochondria was suspended in sucrose (0·25 M)–$MgCl_2$ (15 mM)–ATP (1 mM) at a concentration of 40 mg. of protein/ml. The material was frozen and thawed, treated in the Raytheon sonicator for 2 min. under N_2 and diluted with an equal volume of sucrose (0·25 M). The material was then centrifuged at 15 000g for 6 min. to yield a pellet which was designated fraction DC-1. The supernatant was centrifuged at 105 000g for 45 min., and the pellet obtained was designated fraction DC-2 and the supernatant DC-3.

Fractional precipitation with ethanol. The mitochondrial pellet was suspended in tris–HCl buffer, pH 7·5 (10 mM), and treated in the Raytheon sonicator for 2 min. under N_2. The material was centrifuged at 105 000g for 60 min., the supernatant fluid was taken and ethanol at −10° was slowly added with continuous stirring, with cooling in a bath of ethanol at −15°. As the ethanol concentration was increased the temperature of the material was lowered to −3° at 10% (v/v) ethanol and −5° at 20% (v/v) or more of ethanol, but care was taken that the material did not freeze. At each step in the fractionation precipitates were collected by centrifugation at 10 000g for 10 min. at −5°, and the supernatant was treated with more ethanol until a final concentration of 50% (v/v) was reached. The precipitates obtained at ethanol concentrations of 5, 10, 15, 20, 30, 40 and 50% (v/v) were designated fractions EP-1 to EP-7. The final supernatant was designated fraction EP-8.

Chromatography on triethylaminoethylcellulose. The mitochondria were suspended in tris–HCl buffer, pH 7·5 (10 mM), and were treated for 2 min. in a Raytheon sonicator under N_2. Cold ethanol was then added as described above to give a final concentration of 40% (v/v), and the material was centrifuged at 105 000g for 45 min. The supernatant material was then diluted with 5 vol. of distilled water and dialysed against tris–HCl buffer, pH 7·5 (10 mM), for 16 hr. at 0°. A sample (2 ml.) containing 4 mg. of protein was applied to a triethylaminoethylcellulose (TEAE-cellulose) column (12 cm. × 1·2 cm.) equilibrated with tris–HCl buffer, pH 7·5 (10 mM). The same buffer with a gradient of NaCl increasing in ionic strength from 0 to 1·0 was used for elution (Glomset, 1958), with a total volume of eluate of 1 l., which was collected in 6 ml. fractions. After the E_{280} values of the fractions had been measured, carrier protein was added and protein samples were prepared in the usual way.

Density-equilibrium sedimentation. The pellet of mitochondria obtained after incubation was suspended in 45% (w/w) sucrose solution, at a concentration of 40 mg. of protein/ml. The suspension was treated with ultrasonic vibrations for 3 min. at 0° in the MSE sonicator and was then diluted with an equal volume of 45% sucrose. Then 3 ml. of 61% (w/w) sucrose was added to a Lusteroid centrifuge tube (2·5 cm. × 7·4 cm.), occupying the rounded end of the tube and providing a level base for the sucrose concentration gradient. A portion (5 ml.) of the suspension of

ultrasonically disrupted mitochondria in 45% sucrose was carefully pipetted on top of the 61% sucrose, and then 20 ml. of sucrose solution with a concentration gradient from 45% to 5% (w/w) was run in on top of the mitochondrial suspension, by using a gradient-forming device as described by Britten & Roberts (1960). The final volume in the tube was thus 28 ml.

The tubes containing the gradients were centrifuged at 24 000 rev./min. for 16 hr. in the SW 25 rotor of the Spinco model L centrifuge and the brake was not applied at the end of the run. Fractions from the gradient were collected by puncturing the bottom of the centrifuge tube with a hypodermic needle through which mercury flowed, displacing the sucrose solution upwards and through a tube fitted to a cap on the top of the centrifuge tube. The fractions (0·5–2·0 ml.) were collected in graduated tubes and the volumes measured. Before determining the extinction of the fractions each was made up to a final volume of 3·0 ml., with sodium deoxycholate (final concn. 1%) added to decrease turbidity.

RESULTS

Differential centrifugation. The method of fractionation used yields three fractions: an uncharacterized large-particle fraction, DC-1, which contains 64% of the mitochondrial protein and which probably includes a large proportion of incompletely disrupted mitochondria; a particulate fraction DC-2, which corresponds to the phosphorylating electron-transport particle, ETP_H (Linnane & Ziegler, 1958; Löw & Vallin, 1963), and which contains 14% of the mitochondrial protein; and a supernatant containing 22% of the mitochondrial protein. When this fractionation was carried out on mitochondria that had been incubated with radioactive amino acids, the specific activities of the proteins of the different fractions varied, and changed with the time of incubation (Fig. 1). The rate of incorporation of amino acids into the soluble proteins (fraction DC-3) was much greater than into the other fractions, and after 30 min. of incubation 49% of the radioactivity that was incorporated into protein was in this fraction, with 48% in fraction DC-1 and 3% in fraction DC-2, which showed only a very low rate of incorporation of amino acids into protein. The results shown in Fig. 1 were obtained with a mixture of amino acids (algal-protein hydrolysate), but almost identical results were obtained when leucine was the only radioactive amino acid added. In view of the rapid labelling of the soluble proteins of the mitochondria attempts were made to fractionate this material further and some success was obtained by the use of ethanol precipitation.

Ethanol precipitation. The supernatant proteins obtained after centrifuging the ultrasonically disrupted labelled mitochondria at 105 000*g* for 45 min. were further fractionated by precipitation with increasing concentrations of ethanol at low temperatures. The results of this fractional precipitation are shown in Table 1, which records the amount of protein in each fraction and its specific radioactivity. Most of the soluble mitochondrial proteins were precipitated at ethanol concentrations between 15 and 40% (v/v), but the proteins with the highest specific activity were found in the fraction soluble in 50% (v/v) ethanol, which contained 18% of the soluble proteins of the mitochondria (equivalent to about 4% of the total mitochondrial proteins). Interest therefore focused on this fraction of proteins relatively soluble in ethanol, and some of this material, corresponding

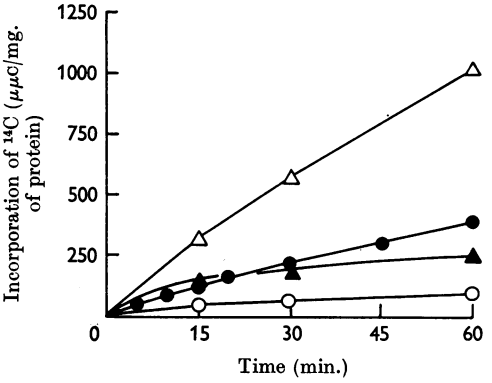

Fig. 1. Course of incorporation of amino acids into components of mitochondria separated by differential centrifugation. Mitochondria were incubated for various times with ^{14}C-labelled algal-protein hydrolysate, disrupted by ultrasonic treatment and fractionated by differential centrifugation, as described in the Materials and Methods section. ●, Intact mitochondria; ▲, fraction DC-1; ○, fraction DC-2; △, fraction DC-3.

Table 1. *Fractional precipitation of mitochondrial soluble proteins by ethanol*

Mitochondria were incubated for 45 min. with ^{14}C-labelled algal-protein hydrolysate, disrupted by ultrasonic treatment and centrifuged at 105 000*g* for 60 min. The supernatant was then treated with an increasing concentration of ethanol as described in the Materials and Methods section.

Fraction	Concn. of ethanol required for precipitation (%, v/v)	Percentage of total soluble protein	Specific activity ($\mu\mu$c/mg. of protein)
EP-1	5	1·2	34
EP-2	10	1·0	34
EP-3	15	2·0	32
EP-4	20	23·1	63
EP-5	30	20·5	51
EP-6	40	22·6	69
EP-7	50	11·1	119
EP-8	Supernatant	18·7	246

to fractions EP-7 and EP-8, was subjected to chromatography on TEAE-cellulose.

Triethylaminoethylcellulose chromatography. Mitochondria labelled by incubation with radioactive amino acid mixture were ultrasonically disrupted and the proteins soluble in 40% (v/v) ethanol were extracted, precipitated by saturation with ammonium sulphate at 0°, and subjected to chromatography on TEAE-cellulose, the proteins being eluted with a salt gradient rising in ionic strength to 1·0. The results (Fig. 2) indicate that a number of protein components, with different specific activities, were present. The fractions with the highest specific activity were those eluted at relatively low ionic strength. It was not possible to characterize these fractions further and the TEAE-cellulose chromatography serves mainly to illustrate the heterogeneity of some of the fractions obtained by ethanol fractionation.

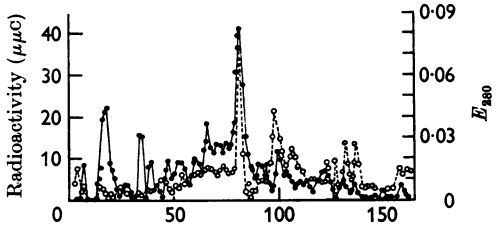

Fig. 2. Chromatography of mitochondrial proteins on TEAE-cellulose. Mitochondria were incubated with ^{14}C-labelled algal-protein hydrolysate, ultrasonically disrupted, and the proteins soluble in 40% (v/v) ethanol were chromatographed on a TEAE-cellulose column in tris–HCl buffer, pH 7·5 (10 mM), the elution being with a concentration gradient of NaCl rising from 0 to 1·0 M. ●, Radioactivity; ○, E_{280}.

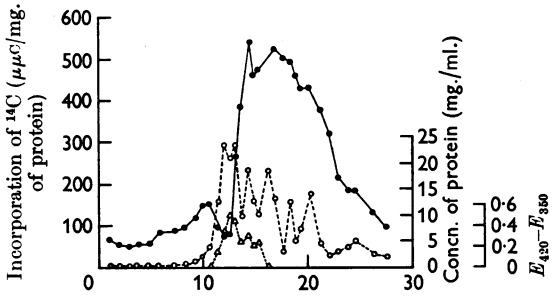

Fig. 3. Fractionation of ultrasonically disrupted mitochondria by density-equilibrium centrifugation. After incubating for 45 min. with DL-[1-^{14}C]leucine, the mitochondria were ultrasonically disrupted and centrifuged in a sucrose density gradient from 61% (ρ 1·30) to 5% (ρ 1·02). ●, Incorporation; ○, concn. of protein; △, $E_{420}-E_{350}$.

Density-equilibrium sedimentation. Mitochondria previously incubated with radioactive leucine were disrupted by ultrasonic vibrations and then centrifuged for 16 hr. in a sucrose density gradient, with a density range from 1·02 to 1·30. After the centrifugation the material had formed a number of distinct layers, and these could be separated when the fractions were collected. The curve of protein concentration in Fig. 3 indicates the separation of different protein species, and the curve showing $E_{420}-E_{350}$, which may be taken as an indication of the position of the cytochromes within the gradient, suggests that there may have been some fractionation of the cytochromes. When the specific activities of the isolated proteins were determined it was found that marked differences occurred between the fractions, the least-active fraction having only 60 μμc/mg. of protein, whereas the most-active fraction had 550 μμc/mg. of protein. The fraction with the highest specific radioactivity was not the lightest fraction, but most of the mitochondrial proteins were found in fractions denser than that with the highest specific activity.

DISCUSSION

Mitochondria are complex structures containing a considerable number of enzymes and other proteins, and Green (1959) has listed 32 enzymes that have been shown to be present in heart mitochondria. Thus there can be no doubt that the protein fractions described in the present paper represent only a few of the protein species present in mitochondria, and it is probable that not one of the fractions isolated represents a single protein component. Despite this shortcoming, the experiments described establish that in isolated mitochondria the rate of incorporation of amino acids into some proteins is much higher than into others. This is most clearly illustrated by Table 1 and Fig. 3.

The lowest rate of amino acid incorporation that was found was into the fraction DC-2 (Fig. 1), which corresponds to the electron-transport particle, ETP_H (Löw & Vallin, 1963). This particle contains succinate dehydrogenase, $NADH_2$-linked cytochrome *c* reductase, cytochrome *a*, cytochrome *b* and cytochrome c_1 (Linnane & Ziegler, 1958; Green & Oda, 1961), and the low rate of incorporation of amino acids into the proteins of this particle suggests that, under the incubation conditions, the mitochondrion is unable to synthesize all of these enzymes, and may not synthesize any of them.

The proteins that show a high rate of incorporation when fractionated by precipitation with ethanol are those with the highest solubility in

ethanol. These may be presumed to be relatively non-polar proteins, possibly lipoproteins. Chromatography on TEAE-cellulose also indicated that the most rapid incorporation was into proteins of relatively low polarity. When the proteins of the mitochondria were fractionated by density-equilibrium centrifugation a number of fractions showed relatively high specific activities and there was a tenfold difference in the specific activities of the isolated proteins. The highest specific activity was in a fraction slightly less dense than most of the mitochondrial proteins and in that region of the gradient which also appeared to contain the cytochromes.

In many ways the results of these experiments agree with those of Roodyn *et al.* (1962), who used rat-liver mitochondria. They too found that the specific activities of different protein fractions varied, and they were unable to show any incorporation of amino acids into two proteins associated with respiratory activities (malate dehydrogenase and cytochrome *c*). The suggestion that lipoproteins may be synthesized in mitochondria finds support in the work of Marsh (1963), who showed that isolated rat-liver mitochondria were capable of incorporating amino acids into both low-density and high-density lipoproteins.

A number of theories have been advanced on the origin of mitochondria, and some of these have been reviewed by Rouiller (1960). There is now more evidence in favour of the view that mitochondria are capable of self-reproduction by fission (Bahr & Zeitler, 1962; Luck, 1963). The relevance of studies on the incorporation of amino acids in isolated mitochondria to the problem of the origin of mitochondria within the cell has been discussed by Roodyn, Reis & Work (1961*b*). The similar results obtained by fractionation of rat-liver mitochondria labelled *in vivo* (Truman, 1963) and *in vitro* (Roodyn *et al.* 1961*a*), in which rapid incorporation was associated with membrane fragments rich in succinoxidase activity, indicates that the incorporation of amino acids into protein by isolated mitochondria is a reflexion of the incorporation *in situ*.

It has been suggested on the basis of experiments with rat-liver mitochondria that the incorporation of amino acids that is observed may represent the synthesis of a relatively small proportion of the mitochondrial protein, to which may be attached other proteins, particularly enzymes, synthesized elsewhere (Truman, 1962). The experiments described above appear to support this view. Lipoproteins may be synthesized by the mitochondria themselves, and respiratory enzymes and cytochromes might be produced by the endoplasmic reticulum or sarcotubular system and then transferred to the lipoprotein skeleton of the mitochondria. The binding of cytochromes and lipids to a protein from ox-heart mitochondria has been described by Criddle, Bock, Green & Tisdale (1962), and though the protein that is most highly labelled in the experiments described above differs from that of Criddle *et al.* (1962) in important ways, it may represent some precursor of the structural protein or be otherwise capable of combining with cytochromes and respiratory enzymes.

SUMMARY

1. Mitochondria labelled by incubation with radioactive amino acids were ultrasonically disrupted and fractionated by differential centrifugation, fractional precipitation with ethanol, chromatography on triethylaminoethylcellulose and density-equilibrium centrifugation.

2. Very slight incorporation of amino acids was found into the proteins of the phosphorylating electron-transport particle.

3. Relatively high incorporation of amino acids was found into proteins soluble in 50% (v/v) ethanol, and in a protein fraction slightly less dense than most of the mitochondrial proteins.

4. It is concluded that the chief site of incorporation of amino acids is a relatively non-polar protein, possibly a lipoprotein, and the implications of this are discussed.

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Biochem. J. (1964) **91**, 64

Transformation of Porphobilinogen into Porphyrins by Preparations from Human Erythrocytes

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The conversion of porphobilinogen into porphyrin has been studied in preparations from various sources, including avian erythrocytes, rabbit reticulocytes, *Rhodopseudomonas spheroides*, *Chlorella* and higher plants. The literature has been reviewed by Rimington (1957, 1958, 1959) and Margoliash (1961). The primary tetrapyrrole formed is uroporphyrinogen. It is as porphyrinogens, rather than as their oxidized derivatives, porphyrins, that uroporphyrinogen and coproporphyrinogen (and presumably those intermediates with seven, six, five and three carboxyl groups/molecule respectively) can be used as substrates for subsequent steps in the biosynthesis of protoporphyrin and haem. The porphyrinogens can be readily oxidized to porphyrins and it is in the porphyrin form that they are detected and estimated.

The synthesis of uroporphyrin III, pseudouroporphyrin [now recognized as heptacarboxylic porphyrin belonging to the III series (Falk, 1955; Lockwood & Davies, 1961; Cornford & Benson, 1963)] and coproporphyrin III, with only traces of protoporphyrin, from δ-aminolaevulic acid by human haemolysates incubated in air was reported by Rimington & Booij (1957). Fallot, Canivet, Mondet & Poidatz (1956) reported that when this system was incubated with δ-aminolaevulic acid in nitrogen uroporphyrin I was formed.

In the present study both crude and partially purified preparations derived from human erythrocytes gave rise to a mixture of porphyrin isomers I and III from porphobilinogen. The amounts and percentages of these isomers were functions of certain incubation conditions. Because of these properties, the preparations seemed ideal material for a study of the enzymic control of uroporphyrinogen III synthesis from porphobilinogen.

MATERIALS

AnalaR (British Drug Houses Ltd.) HCl was found to be 11·6 N (42·3%, w/v). All dilutions were prepared on this basis and are recorded as weight of acid/100 ml. The heparin solution manufactured under the trade name Liquemin by Roche Products Ltd. was used. δ-Aminolaevulic acid was a generous gift from Dr J. Falk, Division of Plant Industry, C.S.I.R.O., Canberra, Australia. Porphobilinogen, kindly supplied by Professor C. Rimington, was derived from human acute-porphyria urines and purified by the method of Cookson & Rimington (1954). Solutions were prepared either in 0·1 M-phosphate buffer, pH 7·4, or in water to which sufficient HCl was added to effect solution of the porphobilinogen. Uroporphyrinogens I and III were prepared from the free porphyrins of the octamethyl esters described by Cornford & Benson (1963), by the procedure of Hoare & Heath (1959). The concentration of uroporphyrinogen was determined as follows: a 0·1 ml. sample was taken, 2 drops of ethanolic 0·2% iodine were added to oxidize it to porphyrin, followed by 2 drops of aq. 0·4% sodium thiosulphate ($Na_2S_2O_3,5H_2O$), and the solution was made up to 20 ml. with 5% (w/v) HCl. The concentration of uroporphyrinogen was calculated by eqn. (A) below.

METHODS

Enzyme preparations

Blood. Except where chicken blood was used for comparison, all preparations were derived from normal adult human blood. The blood (90 ml.) was collected immediately before commencement of enzyme preparations. It was taken, by intravenous puncture, into 4 ml. of 0·9% NaCl