Further Study of Factors Affecting Amino Acid Incorporation into Protein by Isolated Mitochondria

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1. The incorporation of amino acids into protein in isolated mitochondria has now been studied in more detail, and with mitochondria from a range of tissues. 2. Liver mitochondria from newborn rats are twice as active as those from adult rats. 3. The mitochondria are inactivated by excessive homogenization and repeated freezing and thawing. 4. The incorporation is sensitive to conditions of incubation and in particular to the rate of oxygenation, shape of vessel and depth of fluid. Best results are obtained by incubation in flat-bottomed vessels containing suspensions with less than 3 mm. depth of fluid. 5. The requirement for oxidizable substrates has been examined with a range of substances, and most of the common energy-yielding metabolites of the mitochondrion are effective. Their activity is greatly influenced by concentration, and some, but not all, of the substrates show optimum concentrations for incorporation with decreased activity at higher concentrations. 6. Some amino acids can act as energy sources for the incorporation. 7. The effect of increasing the concentration of labelled amino acid is different for different amino acids, and complex effects occur on the addition of amino acid mixtures. 8. It is concluded that the amino acid incorporation into protein finally obtained is the result of many interacting factors related to the structural and metabolic state of the mitochondrion.

Previous publications from this Laboratory have shown that isolated rat-liver mitochondria can incorporate radioactive amino acids into protein, and that the incorporation is not due to bacterial or microsomal contamination (Reis, Coote & Work, 1959; Roodyn, Reis & Work, 1961). The specific enzymes cytochrome *c*, malate dehydrogenase and catalase present in the mitochondrial fraction are not labelled *in vitro* (Roodyn, Suttie & Work, 1962), and the most radioactive proteins found are insoluble lipoproteins associated with the mitochondrial membrane (Roodyn, 1962). The incorporation is stimulated by previous treatment of the intact animal with thyroid hormones and the stimulated particles have sedimentation properties identical with those of mitochondria (Freeman, Roodyn & Tata, 1963; Roodyn, Freeman & Tata, 1965).

In most of the above experiments the methods of isolation and incubation described in detail in Roodyn *et al.* (1961) were used. However, the development of the 'cylinder-plating' method of radioassay (Roodyn *et al.* 1965) has made it possible to investigate with greater ease the important

variables in these procedures. Some of the findings reported by Roodyn *et al.* (1961) have now been studied in greater detail. In particular, the effects of the nature and concentration of the oxidizable substrate and the effect of oxygenation of the mitochondrial suspension have been examined.

METHODS AND MATERIALS

Isolation of subcellular fractions. Mitochondria were isolated by five successive sedimentations at 8000*g* for 10 min. each as described by Roodyn *et al.* (1961, 1965). Unless otherwise stated, the isolation medium was 0.3 M-sucrose–2 mM-EDTA–0.03 M-nicotinamide, pH 7.2.

Oxygen uptake. This was measured manometrically.

Incorporation of radioactive amino acids into protein. Unless otherwise specified mitochondria were incubated at 30° in the medium described by Roodyn *et al.* (1961). It had the following final composition: 0.1 M-sucrose, 0.04 M-KCl, 1.3 mM-EDTA, 0.02 M-nicotinamide, 0.01 M-potassium succinate, 0.016 M-potassium phosphate, 4 mM-AMP, 0.5 mM-NAD, 8 mM-MgSO₄ and 50 µg. of synthetic amino acid mixture/ml. It was adjusted to pH 7.2 and contained 4–6 mg. of mitochondrial protein/ml. Antibiotics were not added. Apart from the experiments in Table 3, most incubations were carried out by the 'cylinder-plating' method described by Roodyn *et al.* (1965). A portion (0.4–0.6 ml.) of a mitochondrial suspension was incubated

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in air at 30° in flat-bottomed specimen bottles (2.2 cm. diam. × 5 cm. long) shaking at 80 cycles/min. After the incubation, cylinders of Whatman no. 3 filter paper (rolled from rectangles 9 cm. × 3 cm.) were dropped into the incubation medium, and the papers were washed with trichloroacetic acid and organic solvents essentially as described by Mans & Novelli (1961). The papers were counted in a scintillation counter as described by Roodyn *et al.* (1965). The counting efficiency for radioactive protein was 70%, so that 1 μC corresponded to 1.6 counts/min. Specific activities in $\mu\text{C}/\text{mg.}$ of protein were calculated from protein N determinations on separate portions of the suspension (Roodyn *et al.* 1965).

Radioactive amino acids. These were obtained from The Radiochemical Centre, Amersham, Bucks. The following were used: L-[U- ^{14}C]valine (6.53 $\mu\text{C}/\mu\text{mole}$), L-[U- ^{14}C]isoleucine (7.30 $\mu\text{C}/\mu\text{mole}$) and DL-[1- ^{14}C]leucine (7.25 $\mu\text{C}/\mu\text{mole}$). The radioactive amino acids were added to the incubation medium at a final concentration of 0.1 $\mu\text{C}/\text{ml.}$ unless otherwise stated. The ^{14}C -labelled algal-protein hydrolysate (Fig. 4) had a specific activity of 1 mc/4.5 mg. and was prepared as described by Roodyn *et al.* (1961).

RESULTS

Source of mitochondrial fractions

The number of tissues used to obtain mitochondrial fractions that exhibit amino acid incorporation into protein *in vitro* has now been extended to

include rat kidney, mouse kidney, mouse liver, frog liver, tadpole liver, rat spleen, mouse spleen, rat lung, rat skeletal muscle and rat heart muscle (Table 1). Dr K. B. Freeman, in this Laboratory, has also obtained incorporation with mitochondria from Krebs II ascites cells. None of these systems have been studied in as great detail as rat-liver mitochondria, and it is possible that they differ in their requirements for optimum incorporation.

No differences were observed in the activity of liver mitochondria from male or female rats. Mitochondria from fed rats appeared in general to have greater activity than those from animals starved for 24 hr., but the results were not very reproducible. Between 6 weeks and 1 year, the age of the rats appeared to have little effect. However, newborn rats gave mitochondrial fractions that were twice as active as those from adult animals. With DL-[1- ^{14}C]leucine as label, the incorporation with mitochondria from 1-day-old rats was 393 $\mu\text{C}/\text{mg.}$ of protein/hr., whereas the corresponding values for 6-, 18- and 52-week-old rats were 206, 210 and 189 $\mu\text{C}/\text{mg.}$ of protein/hr. respectively. [This effect has not yet been studied in detail. In particular, the possible presence of non-mitochondrial structures in the mitochondrial fractions from 1-day-old rats cannot be excluded, since the cell population

Table 1. *Amino acid incorporation in mitochondrial fractions obtained from tissues other than rat liver*

The frog and tadpole livers were obtained from the American bullfrog (*Rana catesbeiana*); premetamorphic tadpoles were used. The skeletal-muscle mitochondria were incubated in the normal medium supplemented with 0.2 mg. of cytochrome *c*/ml. and 1 mg. of bovine serum albumin/ml. Other details are given in the text. The experiments with rat-kidney mitochondria and valine were kindly performed by Dr J. W. Suttie. The mitochondria from frog liver, tadpole liver and skeletal muscle were kindly prepared by Dr J. R. Tata.

Source of mitochondria	Labelled amino acid used	Alteration to medium	Amino acid incorporation into protein ($\mu\text{C}/\text{mg.}$)	
			After 60 min.	After 120 min.
Rat kidney	[^{14}C]Valine	None	74.0	—
	[^{14}C]Valine	Chloramphenicol (10 $\mu\text{g.}/\text{ml.}$) added	31.1	—
	[^{14}C]Valine	Puromycin (10 $\mu\text{g.}/\text{ml.}$) added	7.0	—
	[^{14}C]Valine	Succinate omitted	3.0	—
	[^{14}C]Isoleucine	None	115.5	119.9
Mouse kidney	[^{14}C]Isoleucine	Glucose as energy source	16.2	—
	[^{14}C]Isoleucine	None	149.7	268.0
Mouse liver	[^{14}C]Isoleucine	None	75.2	160.0
Frog liver	[^{14}C]Valine	None	58.2	—
Tadpole liver	[^{14}C]Valine	None	213.6	—
	[^{14}C]Isoleucine	None	93.0	—
Rat spleen	[^{14}C]Isoleucine	None	85.1	101.3
	[^{14}C]Isoleucine	Glucose as energy source	61.9	—
Mouse spleen	[^{14}C]Isoleucine	None	101.0	289.0
Rat lung	[^{14}C]Isoleucine	None	32.5	44.1
Rat skeletal muscle	[^{14}C]Isoleucine	None	21.7	—
Rat heart muscle	[^{14}C]Isoleucine	None	24.2	—

and morphology of the liver cells change profoundly during the first week after birth (Dadoune, 1963).] The most striking effect of the physiological status of the animal on mitochondrial incorporation is that of thyroid hormones. This effect is described in detail by Roodyn *et al.* (1965).

Apart from the above results, all the experiments below were done with liver mitochondria isolated from normal 100g. hooded (Mill Hill) rats. The animals were not deprived of food before the experiment.

Method of isolation

Method of homogenization. There was little difference in the final activity whether the homogenate was prepared by short or prolonged treatment in the Potter-Elvehjem homogenizer, or even by short treatment in the Waring Blendor. Presumably, the whole homogenate must have exerted some protective effect, since similar treatment of the mitochondria, once isolated, resulted in inactivation (Expts. 1 and 2 in Table 2). The sensitivity of the isolated mitochondria to structural damage was confirmed by showing inhibition after repeated freezing and thawing (Expt. 3 in Table 2).

Isolation medium. The effect of the composition of the isolation medium has been studied in more detail. The optimum concentration of sucrose is approx. 0.3M (Roodyn *et al.* 1965). Most of the present experiments were with the medium originally devised by Reis *et al.* (1959), i.e. 0.3M-sucrose-0.03M-nicotinamide-2mM-EDTA, pH 7.2. How-

ever, I have not been able to obtain a reproducible improvement in incorporation with nicotinamide in recent experiments with improved conditions of incubation. The reason for this variability is at present unclear and it is not possible to say definitely whether nicotinamide is beneficial. The effect of EDTA was also somewhat variable, but on averaging three experiments the addition of EDTA (final concn. between 1 and 5mM) resulted in a 30% improvement in incorporation. It is probably best to retain EDTA as a precaution against contamination with heavy-metal ions and ribosomes, since these dissociate in EDTA.

Changes in the pH of the isolation medium in the range pH 6.5-7.8 did not have more than a 20% effect on the initial rate of incorporation, although the incorporation system seemed somewhat more stable after isolation at pH 6.5 than at pH 7.8. However, because of the increased danger of precipitation of microsomal material into the mitochondrial fraction at lower pH values, pH 7.2 was generally employed. It may be noted that changes in the pH of the incubation medium have a much greater effect (see below).

Since it was possible that a medium that was optimum for amino acid incorporation might also preserve mitochondrial integrity during isolation, mitochondria were isolated in the normal (non-radioactive) incubation medium. However, the activity of the fraction was similar to that isolated in sucrose-EDTA-nicotinamide medium.

Centrifugal procedures. The range of centrifugal forces in which the incorporation system sediments

Table 2. *Effect of various physical treatments on amino acid incorporation in rat-liver mitochondria*

[¹⁴C]Isoleucine was used in Expt. 1 and [¹⁴C]valine in Expts. 2 and 3. In Expt. 2 the original homogenate had been prepared by 30 sec. treatment in the Potter-Elvehjem homogenizer and the final mitochondrial suspension contained 13.6 mg. of protein/ml. Other details are given in the text.

	Amino acid incorporation into protein ($\mu\text{C}/\text{mg.}$)		
	After 30 min.	After 60 min.	After 120 min.
Expt. 1. Conditions of homogenization of liver			
Potter-Elvehjem homogenizer (30 sec.)	—	97.3	130.0
Potter-Elvehjem homogenizer (2 min.)	—	116.6	131.0
Waring Blendor (2 min.)	—	94.0	146.6
Expt. 2. Treatment of isolated mitochondria			
Potter-Elvehjem homogenizer (30 sec.)	19.7	39.0	59.6
Potter-Elvehjem homogenizer (1 min.)	12.2	36.9	58.8
Potter-Elvehjem homogenizer (3 min.)	7.2	17.9	33.5
Waring Blendor (3 min.)	2.5	3.5	3.3
Expt. 3. Effect of freezing and thawing in acetone-solid CO ₂			
No treatment	—	52.7	—
Frozen and thawed once	—	25.0	—
Frozen and thawed five times	—	7.7	—

Fig. 1. Centrifugal procedure required to sediment the mitochondrial amino acid-incorporation system. Rat-liver fractions were incubated with [¹⁴C]isoleucine. Other details are given in the text. The centrifugal procedure required to sediment 50% of the activity is 40000g-min. (5 min. at 8000g).

is given on a logarithmic basis in Fig. 1, which shows that 40000g-min. (8000g for 5 min.) would sediment half the activity. Within the range shown in Fig. 1, the specific radioactivity of mitochondrial protein does not vary greatly (Roodyn *et al.* 1965), so that easily sedimented mitochondria are as active on a protein basis as less easily sedimented mitochondria. Little or no incorporation is obtained above or below the range shown in Fig. 1, since nuclei and microsomal fractions are relatively inactive under the conditions used to demonstrate mitochondrial incorporation (Roodyn *et al.* 1965).

Repeated washing by sedimentation and suspension appears to have little effect on the incorporation (Roodyn *et al.* 1961), although microsomal contamination, as judged by RNA and glucose 6-phosphatase, is drastically decreased. The repeated washing also decreased the rate of endogenous respiration from 17.2 μ l. of oxygen/mg. of protein/hr. to 7.3 μ l. of oxygen/mg. of protein/hr., i.e. by about 60%. This observation may explain the stimulatory effect of added oxidizable substrates (see the Discussion section). The ability of mitochondria to withstand the rather rigorous washing procedure suggests that the system is not too sensitive to mild physical treatments. Mitochondria have been stored as a pellet at 0° for 24 hr. without any serious loss in activity. Rigorous temperature control during the isolation is apparently not essential, since mitochondria isolated at 15° rather than 0° still retain 75–95% of their activity. More violent treatments, however, e.g. excessive homogenization, freezing and thawing (Table 2) or lysis

with the neutral detergent Triton X-100 (Roodyn *et al.* 1965), are inhibitory.

Conditions of incubation

The most profound effects on the incorporation system are seen when the conditions of incubation are altered.

Temperature. Best results were obtained at 30° or 37° but at the higher temperature some clumping occurred. The preferable temperature is therefore 30°. There was negligible incorporation at 0°, which confirmed that there was little non-specific adsorption of radioactive amino acid on mitochondrial protein. (It is the custom in this work to subtract the zero-time value from all results, to eliminate artifacts due to adsorption. The absence of incorporation at 0° also eliminates any non-specific adsorption, which may take 30–60 min. to occur.) Incorporation at 25° was about 60% of that observed at 30°.

Rate of oxygenation and shape of vessel. Most of the previous experiments in this Laboratory were carried out under oxygen in 50 ml. conical flasks fitted with gassing heads, and 3–6 ml. of mitochondrial suspension plus incubation medium was used (Reis *et al.* 1959; Roodyn *et al.* 1961). The optimum mitochondrial concentration was found to be about 5 mg. of protein/ml., and under these conditions oxygen clearly stimulated incorporation. However, it was observed later that, if the amount of fluid in the flasks was decreased from 3–6 ml. to 1 ml. or less, the incorporation observed in air increased considerably, and the percentage stimulation by oxygen was greatly decreased. Incubation in a variety of vessels with different depths of fluid and conditions of oxygenation was then examined and it was found that these factors had an important influence on the incorporation obtained (Table 3). In every case decrease in the depth of fluid not only increased the incorporation in air but also decreased the stimulatory effect of oxygen. With less than 2 mm. of fluid oxygen had little effect, and it was therefore possible to carry out the incubations in 22 mm. diam. specimen bottles with 0.4–0.6 ml. of fluid. The development of the 'cylinder-plating' method of incubation and radio-assay (Roodyn *et al.* 1965) greatly simplified the experimental procedure for studying amino acid incorporation *in vitro*.

The effects observed were due to inadequate oxygenation and not to accumulation of carbon dioxide, since incubation in Warburg vessels with or without a trap for carbon dioxide gave similar results.

Effect of pH. The incorporation is more sensitive to changes in pH of the incubation medium than of the isolation medium, and a definite pH optimum is

Table 3. *Effect of type of incubation vessel, rate of oxygenation and depth of fluid on amino acid incorporation in rat-liver mitochondria*

The rate of shaking was 80 cycles/min. Expts. 1 and 2 were with [^{14}C]valine and Expts. 3 and 4 were with [^{14}C]isoleucine. Other details are given in the text. Values in parentheses are ratios of incorporation in O_2 to incorporation in air.

	Incubation vessel	Vol. of fluid (ml.)	Depth of fluid (mm.)	Gas phase	Amino acid incorporation into protein ($\mu\text{C}/\text{mg}/\text{hr.}$)
Expt. 1	5 mm. diam. tube	0.1	6	Air	69.7
		0.2	12	Air	59.0
		0.3	18	Air	49.8
		0.4	24	Air	33.7
		0.5	30	Air	25.7
Expt. 2	15 mm. diam. tube	1.5	12	O_2 (6 ml./min.)	14.2
		1.5	12	O_2 (300 ml./min.)	58.8
Expt. 3	15 mm. diam. tube	1.5	12	Air	7.8
		1.5	12	O_2	13.6 (1.74)
	50 ml. flask	1.5	2	Air	32.2
		1.5	2	O_2	36.4 (1.13)
	Manometer flask	1.5	5	Air	28.6
		1.5	5	O_2	33.4 (1.16)
		6.0	10	Air	5.8
		6.0	10	O_2	20.5 (2.82)
Expt. 4	Specimen bottle (22 mm. diam.)	1.0	3	Air	84.2
		1.0	3	O_2	118.0 (1.40)
		3.0	6	Air	22.8
		3.0	6	O_2	87.0 (3.81)

observed near pH 7.2. The rates of incorporation observed at pH 6.5 and pH 7.8 were about 30 and 50% respectively of that observed at pH 7.2.

Attempted improvements to incubation medium. In most of the work published from this Laboratory, the incubation medium developed initially by Reis *et al.* (1959) and given in detail as medium B by Roodyn *et al.* (1961) has been used. Many variations in the composition of this medium have been tried, but none have produced striking improvements. With complex media the systematic investigation of every possible combination of substances, at different concentrations, was impracticable, even with the improved method of assay. Various combinations were omitted or their amounts altered, but in every case alteration to the medium resulted in less activity than in the control.

Apart from these modifications, attempts were made to improve the incorporation by additions to the medium. In confirmation of previous observations (Reis *et al.* 1959; Roodyn *et al.* 1961), cell sap had no effect. A mitochondrial extract prepared by the method of Truman & Korner (1962b) was also without effect. This suggests that the failure to obtain stimulation with cell sap was not due to the

Fig. 2. Effect of preincubation in the absence of oxidizable substrate on amino acid incorporation into protein in rat-liver mitochondria. Mitochondria were incubated with [^{14}C]valine under O_2 in the normal medium from which succinate had been omitted. Potassium succinate (final concn. 10 mM) was added at the following times: A, 0 min.; B, 5 min.; C, 10 min.; D, 15 min.; E, 30 min.; F, 60 min.; G, 90 min. Radioactive amino acid was added at the beginning of the experiment to all vessels. Other details are given in the text.

absence of specific mitochondrial soluble factors necessary for incorporation. A range of concentrations (0.1–1.0 mg./ml.) of bovine serum albumin or cytochrome *c* also had no effect. The incorporation system was not seriously affected by 10 mM-mercaptoethanol added either to the incubation or to the isolation media. As with all negative results, failure to observe an effect may well have been due to unsuitable experimental conditions.

Role of oxidizable substrates. Apart from a definite requirement for AMP, inorganic phosphate and Mg^{2+} ions, there is a clear dependence of the system on an oxidizable substrate (Roodyn *et al.* 1961). In addition, incubation in the absence of an energy source irreversibly inhibits the incorporation system (Roodyn *et al.* 1961). This effect has now been studied in more detail and definite inhibition is observed after only 10 min. in the absence of succinate (Fig. 2). Incubation for only 30 min. results in almost total inhibition of the system. Some effect on oxygen consumption is observed at this time (Fig. 3), since the initial rate of succinate oxidation after adding substrate is increased, probably owing to loosening of respiratory control. More prolonged incubation of mitochondria in the

absence of oxidizable substrate (e.g. 60–90 min.) results in inhibition of oxygen uptake. Preliminary experiments have indicated that the incorporation system is more sensitive to incubation in the absence of an energy source than is oxidative phosphorylation.

After the essential requirement of the incorporation for an oxidizable substrate had been confirmed, the number of such substrates studied was extended from succinate and α -oxoglutarate (Roodyn *et al.* 1961) to the list given in Table 4. Most of the normal oxidizable metabolites of the mitochondrion can act as an energy source, although some are more effective than others. With the substrate concentration 10 mM, succinate and α -oxoglutarate are the most effective of the tricarboxylic acid-cycle intermediates. It is important to note that certain amino acids (e.g. serine, glutamate, glutamine) are suitable energy sources. Rather low activity was observed with alanine and aspartate (see also Figs. 8 and 9). Pyruvate, β -hydroxybutyrate and hexanoate appeared to be relatively poor substrates, and glycerol 1-phosphate was moderately active; pyruvate plus malate and hexanoate plus malate are more effective, however (see Figs. 5 and 6). A mixture of several substrates was more active than succinate, and it is possible that the incorporation system could be considerably improved by using various complex mixtures of oxidizable substrates.

The values for the relative efficiencies of the various substrates given in Table 4 are, unfortunately, deceptive, because it was found that the oxidizable substrates gave very different results at different substrate concentrations. These results are given in detail in Figs. 4–9, with the values obtained in the same experiments with 10 mM-succinate. Most of the substrates fall into three classes as follows: (1) those showing a clear plateau-like curve with little or no falling off in activity at higher concentrations (succinate, hexanoate, hexanoate plus malate); (2) those showing some decrease of activity at higher concentrations (malate, pyruvate, pyruvate plus malate, fumarate); (3) those showing a marked fall in activity at higher concentrations, and a clear optimum at 5 mM or less (oxaloacetate, α -oxoglutarate, *cis*-aconitate, citrate). In addition to these differences, Figs. 4–9 show that the stability of the incorporation is not the same in all cases. For example, in Fig. 8, the results with 10 mM-*cis*-aconitate after 30 min. were similar to those after 60 min. However, in the same experiment, the incorporation after 60 min. in the presence of 10 mM-succinate was almost 50% greater than that after 30 min. Similar differences may be observed between hexanoate plus malate and succinate in Fig. 6, and malate and succinate in Fig. 5. In the latter experiment, incorporation was more prolonged with malate

Fig. 3. Effect of preincubation in the absence of oxidizable substrate on O_2 uptake by rat-liver mitochondria. Mitochondria were incubated in air in the medium used for amino acid incorporation, but with succinate omitted. Potassium succinate (final concn. 10 mM) was added at the following times (shown by the arrows): A, 0 min.; B, 30 min.; C, 60 min.; D, 90 min.; E, 120 min. Other details are given in the text.

Table 4. *Relative efficiency of various substrates as an energy source for amino acid incorporation in rat-liver mitochondria*

Apart from glutamate, which had a concentration of $4\mu\text{moles/ml.}$, all substrates were present at a concentration of $10\mu\text{moles/ml.}$ The 'average relative incorporation' is the average value of the incorporation observed with a given substrate divided by that observed with succinate. The 'substrate mixture' contained (per ml.) $1.25\mu\text{moles}$ each of succinate, glucose, fumarate, hexanoate, α -oxoglutarate, β -hydroxybutyrate, malate and citrate. Other details are given in the text.

Substrate	Amino acid incorporation into protein ($\mu\mu\text{C}/\text{mg.}/\text{hr.}$)			Average relative incorporation
	With DL- ^{14}C leucine	With ^{14}C isoleucine	With ^{14}C valine	
Tricarboxylic acid-cycle intermediates				
Oxaloacetate	55.5	—	—	0.44
Citrate	37.5	—	—	0.59
cis-Aconitate	50.6	—	—	0.40
α -Oxoglutarate	—	80.0	95.0	0.99
Succinate	126.9	77.0	99.0	1.00
Fumarate	61.5	51.4	43.5	0.53
Malate	104.5	40.8	103.0	0.76
Amino acids				
Alanine	8.9	13.1	—	0.12
Aspartate	13.9	—	—	0.11
Glutamate	—	58.0	—	0.75
Glutamine	—	93.0	—	1.21
Serine	—	33.7	—	0.44
Other substances				
Pyruvate	46.3	—	8.0	0.23
β -Hydroxybutyrate	34.8	21.8	1.0	0.19
Hexanoate	16.8	10.2	2.0	0.09
Glycerol 1-phosphate	49.9	—	—	0.39
Substrate mixture	—	104.0	—	1.35

Fig. 4. Effects of succinate, fumarate and α -oxoglutarate as energy sources for amino acid incorporation into protein in rat-liver mitochondria. O, Succinate (60 min. incubation); $\blacktriangle$, fumarate (30 min. incubation); $\triangle$, fumarate (60 min. incubation); $\square$, α -oxoglutarate (60 min. incubation). The experiments with succinate and fumarate were with [^{14}C]leucine; that with α -oxoglutarate was with ^{14}C -labelled algal-protein hydrolysate ($0.33\mu\text{c/ml.}$). Other details are given in the text. The broken lines give the values after 30 min. (A) and 60 min. (B) incubation with 10mM -succinate.

than with succinate. The final amount of radioactivity obtained in the mitochondrial protein will therefore depend on the nature of the oxidizable substrate, its concentration and the time of incubation.

Effect of amino acid concentration. Previously, with ^{14}C -labelled algal-protein hydrolysates and synthetic amino acid mixtures (Roodyn *et al.* 1961), it was concluded that the increased incorporation observed on adding more amino acid was due to an increase in the specific radioactivity in the free amino acids of the pool and not to a true stimulation of protein synthesis. However, by studying individual amino acids, it now appears that this is an oversimplification. Three experiments were carried out as follows (Table 5). In the first, the concentration of labelled amino acid was increased, but that of the synthetic amino acid mixture was kept constant. With valine, a tenfold increase in concentration had no effect on the incorporation. It may be concluded that the pool of endogenous valine is readily saturated. However, a similar increase with leucine resulted in doubling of the incorporation. The incorporation system is thus more easily saturated with valine than with leucine. The effect observed with a mixture of

Fig. 5. Effects of malate, pyruvate and pyruvate plus malate as energy sources for amino acid incorporation into protein in rat-liver mitochondria. ●, Malate (30 min. incubation); ○, malate (60 min. incubation); ■, pyruvate (30 min. incubation); ▲, pyruvate+mm-malate (30 min. incubation); △, pyruvate+mm-malate (60 min. incubation). [^{14}C]Leucine was used as label in all experiments. Other details are given in the text. The broken lines give the values after 30 min. (A) and 60 min. (B) incubation with 10 mM-succinate.

Fig. 6. Effects of hexanoate and hexanoate plus malate as energy sources for amino acid incorporation into protein in rat-liver mitochondria. ▲, Hexanoate (30 min. incubation); △, hexanoate (60 min. incubation); ●, hexanoate+mm-malate (30 min. incubation); ○, hexanoate+mm-malate (60 min. incubation). [^{14}C]Leucine was used as label in all experiments. Other details are given in the text. The broken lines give the values after 30 min. (A) and 60 min. (B) incubation with 10 mM-succinate.

labelled amino acids must therefore now be considered in terms of the behaviour of all the individual amino acids present.

In the second experiment given in Table 5 the concentration of labelled amino acid (valine) was kept constant, and the concentration of synthetic amino acid mixture (not containing valine) increased from 0.063 to 1.25 $\mu\text{moles/ml}$. This resulted in doubling of the incorporation. Since the amount of valine was constant, the effect could not be due to labelling of the pool, and at first sight may be interpreted as demonstrating a requirement of the incorporation system for amino acids. However, it is also likely that some of the amino acids present can act as oxidizable substrates, either directly or after conversion into oxidizable α -oxo acids (see Table 4).

In the third experiment given in Table 5 (with isoleucine) the concentrations of both the labelled amino acid and the synthetic amino acid mixture were increased. A marked stimulation was obtained, and there appeared to be optimum concentrations

for incorporation (0.04 μmole of isoleucine/ml. and 0.63 μmole of amino acid mixture/ml.). In this experiment, the direct effects on labelling of the pool and the indirect effect of the amino acid mixture act simultaneously. The various amino acids therefore behave differently with regard to saturation of the incorporation system, and also interact with each other in a complex way.

Once labelled with radioactive amino acid, the protein does not lose its radioactivity even after nearly 2 hr. incubation in the presence of excess of non-radioactive amino acid (Fig. 10). This shows that the incorporation cannot be due to rapid reversible exchange reactions between protein and free amino acids.

DISCUSSION

The number of mitochondrial fractions that have been reported capable of incorporating radioactive amino acids into protein has now increased considerably. They include mitochondria from adult

Fig. 7. Effects of β -hydroxybutyrate and oxaloacetate as energy sources for amino acid incorporation into protein in rat-liver mitochondria. ▲, β -Hydroxybutyrate (30 min. incubation); △, β -hydroxybutyrate (60 min. incubation); ●, oxaloacetate (30 min. incubation); ○, oxaloacetate (60 min. incubation). [14 C]Leucine was used as label in all experiments. Other details are given in the text. The broken lines give the values after 30 min. (A) and 60 min. (B) incubation with 10 mM-succinate.

Fig. 8. Effects of *cis*-aconitate and aspartate as energy sources for amino acid incorporation into protein in rat-liver mitochondria. ●, *cis*-Aconitate (30 min. incubation); ○, *cis*-aconitate (60 min. incubation); ▲, aspartate (30 min. incubation); △, aspartate (60 min. incubation). [14 C]Leucine was used as label in all experiments. Other details are given in the text. The broken lines give the values after 30 min. (A) and 60 min. (B) incubation with 10 mM-succinate.

rat liver (McLean, Cohn, Brandt & Simpson, 1958; Reis *et al.* 1959; Greengard & Campbell, 1959; Korner, 1959; Rendi, 1959; Roodyn *et al.* 1961; Truman & Korner, 1962a; Braun, Marsh & Drabkin, 1963; Marsh, 1963; Bronk, 1963; Kroon, 1964; Grief & Kandemir, 1965), regenerating rat liver (Braun *et al.* 1963; Dr K. B. Freeman, personal communication), 1-day-old rat liver (this paper), rat tumour (Greengard & Campbell, 1959), mouse, frog and tadpole liver (this paper), ox heart (Kroon, 1963a, 1964), calf heart (Simpson, Skinner & Lucas, 1961), rat heart (Kalf, 1963; this paper), sheep thyroid (Singh, Raghupathy & Chaikoff, 1964), insect muscle (Neupert, 1965), rat skeletal muscle (McLean *et al.* 1958; this paper), rat and mouse spleen, rat and mouse kidney, rat lung (this paper), Krebs II ascites cells (Dr K. B. Freeman, personal communication), bean hypocotyl (Webster, 1954), seedlings from *Vigna sinensis* (Das, Chatterjee & Roy, 1964), the sea urchin *Paracentrotus lividus* (Giudice, 1960) and *Tetrahymena pyriformis* (Mager, 1960). Although not all these mitochondrial fractions have been characterized in detail, and the contribution of non-mitochondrial contamination

has not been eliminated in every case, it is probably reasonable to assume that the ability to incorporate amino acids into protein *in vitro* is a universal property of mitochondria.

The sensitivity of the system to the rate of oxygenation, shape of incubation vessel and depth of fluid (Table 5) probably explains much of the day-to-day variability in results encountered when flasks fitted with gassing heads are used (Roodyn *et al.* 1961). It also makes precise comparison between the results of different workers difficult, since exact conditions of incubation are rarely given. Usually incubations are carried out in test tubes or centrifuge tubes (Truman & Korner, 1962a; Kroon, 1963a) containing about 1 ml. of fluid. Under these conditions the incorporation would certainly be affected by the diameter of the tube, the rate of shaking and the angle at which the tube was clamped. The response of the incorporation system to different oxygen concentrations may have some significance in the living cell as a

Fig. 9. Effects of citrate and alanine as energy sources for amino acid incorporation into protein in rat-liver mitochondria. ●, Citrate (30 min. incubation); ○, citrate (60 min. incubation); ▲, alanine (30 min. incubation); △, alanine (60 min. incubation). [^{14}C]Leucine was used as label in all experiments. Other details are given in the text. The broken lines give the values after 30 min. (A) and 60 min. (B) incubation with 10 mm-succinate.

mechanism of control of mitochondrial protein synthesis and replication (Roodyn, 1965).

The most important difference between the results in the present paper and those of some other workers is in the response to added oxidizable substrate. It has been found consistently in this Laboratory that very low rates of incorporation are obtained in the absence of an oxidizable substrate (Roodyn *et al.* 1961; Figs. 4–9 in this paper). The system is irreversibly damaged by incubation for a relatively short time in the absence of oxidizable substrate (Roodyn *et al.* 1961; Fig. 2 in this paper) and thus appears to require a continuous supply of oxidative energy for its integrity. Dependence on an oxidizable substrate has also been shown by Webster (1954), McLean *et al.* (1958) and Kalf (1963). However, Truman & Löw (1963) obtained amino acid incorporation with isolated ox-heart mitochondria in a medium not containing oxidizable substrate. Also, the addition of succinate or glutamate (10 mm) to their system resulted in substantial inhibition. (The system also showed no requirement for Mg^{2+} ions and no phosphate was added.) The addition of succinate also decreased the radioactivity of the acid-soluble pool of amino

acids, and these authors suggested that this was due to stimulation of transamination and oxidation of amino acids. The fall in incorporation was ascribed to a decrease in the availability of amino acids for protein synthesis.

Similar observations were made by Kroon (1963b). Rates of incorporation, about three times those observed in this Laboratory, were obtained with rat-liver mitochondria in a medium containing tris, potassium chloride, magnesium chloride, EDTA, phosphate, ADP and synthetic amino acid mixture, but again with no oxidizable substrate. The addition of isocitrate or α -oxoglutarate (20 mm) again resulted in a substantial decrease in the activity. Since the effect was reversed by adding ammonium chloride, it was suggested that the high NADPH/NAD $^{+}$ ratio during isocitrate oxidation was lowered by NH_4^{+} ions and that optimum incorporation is obtained with low concentrations of NADPH. In a more detailed study, using both rat-liver mitochondria and ox-heart mitochondria, Kroon (1964) demonstrated inhibition under similar conditions with α -oxoglutarate, β -hydroxybutyrate, glutamate, pyruvate plus malate, succinate and isocitrate.

Although there is no obvious explanation for the differences between the results in the present paper and those of Truman & Löw (1963), there are some indications for the reasons for the apparent contradiction between my results and those of Kroon (1963b, 1964). With certain substrates I have observed greatly decreased incorporation at concentrations of 20 mm, and indeed Kroon (1964) used 20–30 mm-substrate to demonstrate his effects. The substrates exhibiting this effect most clearly were oxaloacetate, α -oxoglutarate, *cis*-aconitate and citrate. Thus we are in agreement that concentrations of substrates in the range 20–30 mm can apparently inhibit. The differences in results with lower substrate concentrations (i.e. 5–10 mm) may be ascribed to differences in the method of preparation of mitochondria. Kroon (1963a,b, 1964) used unwashed mitochondria that had been preincubated with ribonuclease to inactivate microsomal contamination. Since the extensive washing used in our method greatly decreased the endogenous respiration, much of the pool of endogenous substrates in the mitochondrion has been removed. Under such conditions, it is likely that there would be low incorporation in the absence of substrate, and stimulation on addition. In unwashed mitochondria, however, containing adequate oxidizable substrate, the addition of substrate to give a 5 mm concentration may well have no stimulatory effect.

Certain amino acids can act as an energy source (Table 4). Also, the α -oxo acids that are related to alanine, aspartate and glutamate, i.e. pyruvate, oxaloacetate and α -oxoglutarate, can act as

Table 5. *Effect of concentration of added amino acids on amino acid incorporation in rat-liver mitochondria*

The amino acid incorporation was calculated from the specific radioactivity of the added amino acid and the radioactivity of the protein. No correction was made for any endogenous amino acid present. The synthetic amino acid mixture contained (per μ mole) the following amounts of amino acids ($m\mu$ moles): alanine, 107; arginine, 13; aspartate, 53; asparagine, 27; cystine, 53; glutamate, 107; glutamine, 80; glycine, 107; histidine, 27; isoleucine, 40; leucine, 40; lysine, 53; methionine, 53; phenylalanine, 13; proline, 27; serine, 80; threonine, 53; tryptophan, 13; valine, 53 (valine was omitted in Expt. 2). Other details are given in the text.

	Labelled amino acid used	Concn. of added amino acids (μ moles/ml.)		Amino acid incorporation into protein (uncorrected for pool) ($m\mu$ moles/g./hr.)
		Amino acid used as label	Synthetic amino acid mixture	
Expt. 1 (label increased, mixture constant)	$[^{14}\text{C}]$ Valine	0.034	0.370	22.6
		0.172	0.370	21.0
		0.325	0.370	20.7
	$[^{14}\text{C}]$ Leucine	0.028	0.370	13.0
		0.359	0.370	26.6
Expt. 2 (label constant, mixture increased)	$[^{14}\text{C}]$ Valine	0.020	0.063	6.9
		0.020	0.313	9.6
		0.020	0.626	11.0
		0.020	0.939	14.2
		0.020	1.250	15.0
Expt. 3 (label and mixture increased)	$[^{14}\text{C}]$ Isoleucine	0.018	0	16.4
		0.020	0.063	24.5
		0.030	0.313	38.4
		0.042	0.626	56.7
		0.054	0.939	55.3
		0.066	1.250	40.2

Fig. 10. Effect of adding non-radioactive amino acid during the incubation on the amino acid incorporation into protein in rat-liver mitochondria. Mitochondria were incubated with 0.0154μ mole (0.1μ c) of $[^{14}\text{C}]$ valine/ml. At the arrows, unlabelled valine (1μ mole/ml.) was added. Other details are given in the text.

oxidizable substrates and have different effects at different concentrations (Figs. 4, 5 and 7). Also, there are differences in the response of the incorporation system to changes in the concentration of different labelled amino acids (Table 5). These observations emphasize the complexity of the effect of adding a mixture of amino acids.

Little is known about the transport of amino acids across the mitochondrial membrane, and in particular it is not certain whether the uptake is active or passive (Garfinkel, 1963). If active transport of amino acids does occur it may exert an important controlling influence over amino acid incorporation into protein. Damage to the membrane could either destroy the active transport process, or induce leakiness, with resultant loss of amino acids from the intramitochondrial pool into the extramitochondrial fluid. Once taken up, the radioactive amino acid must equilibrate with the intramitochondrial pool of amino acids. There is good evidence that the mitochondrion contains a significant pool of free amino acids. For rat-liver mitochondria Truman & Korner (1962a) give a value of 4.50μ g. of amino N/mg. of protein, and Roodyn *et al.* (1961) report 3.3μ g. of amino N/mg.

of protein. These values correspond to about 30–40 μg . of amino acid/mg. of protein, or 3–4% of the protein. All the commonly occurring amino acids are present in the pool in significant amounts, apart from proline and cysteine (Truman & Korner, 1962a; Baird, 1964). The radioactive amino acid will therefore be diluted by the unlabelled pool. Unfortunately, the amount of free amino acid increases during incubation, almost certainly because of the action of proteolytic enzymes (Alberti & Bartley, 1963; Baird, 1964). This effect may result in an apparent decrease in incorporation by dilution of the label during the experiment.

If the amino acid can undergo transamination with other α -oxo acids, its concentration in the pool will be determined by the concentrations of these acids, as well as by the concentration of other amino acids competing for the transaminase sites. Indeed, the concentrations of certain mitochondrial amino acids (such as glutamate and aspartate) are influenced by complex energy-transfer reactions in the mitochondrion (Slater & Tager, 1963; Tager & Haan, 1964). The concentrations of free amino acid in certain cases may therefore be intimately related to the metabolic state of the mitochondrion. The activation of the amino acid and its insertion into a peptide chain are energy-requiring processes and would therefore be influenced by the availability of respiratory energy. The concentrations of oxidizable substrates, Mg^{2+} ion, adenine nucleotides and inorganic phosphate will therefore all profoundly influence these processes. These concentrations will be determined by the overall metabolic and structural state of the mitochondrion, as will the composition of the amino acid pool. The entire process of amino acid incorporation into mitochondrial protein therefore depends on complex interactions within the mitochondrion. It may therefore be a useful supplementary tool in the current rapidly converging studies of the mitochondrion in the fields of oxidative phosphorylation, ion transport, swelling and membrane structure (Lehninger, 1964).

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