Importance of the Osmolarity of the Incubation Medium on Amino Acid Incorporation into Protein by Isolated Rat Liver Mitochondria

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1. Incorporation of [^{14}C]leucine into protein by isolated rat liver mitochondria was examined by using incubation media similar to those used by Sandell, Löw & Decken (1967) (medium A) and Roodyn, Reis & Work (1961) (medium B). The incorporation process was found to be almost completely inhibited in medium A. 2. By decreasing the amount of sucrose and omitting tris-hydrochloric acid from medium A, incorporation proceeded at a rate higher than that found in medium B. It was found that the inhibitory action of medium A was due to its high osmolarity. 3. Oxidative phosphorylation and RNA synthesis by the isolated mitochondria proceeded at the same rate in media essentially the same as media A and B. 4. There was a partial inhibitory action of medium A on leucine uptake by the mitochondria and also on the formation of leucyl-transfer-RNA. The major block of inhibition by the hyperosmolarity of medium A seemed to be located at a later step of protein synthesis involving mitochondrial ribosomes. 5. Protein synthesis by *Escherichia coli* B was only slightly inhibited, if at all, in hyperosmotic media in which protein synthesis by isolated mitochondria was completely stopped.

Incorporation of radioactive amino acids into protein by isolated rat liver mitochondria has been reported by various workers (McLean, Cohn, Brandt & Simpson, 1958; Roodyn, Reis & Work, 1961; Truman & Korner, 1962; Kroon, 1963). It has also been shown that the incorporation is mainly into insoluble, perhaps structural, mitochondrial protein (Roodyn, 1962; Haldar, Freeman & Work, 1966), and at least some of the specific soluble proteins such as cytochrome *c* and malate dehydrogenase are not synthesized *in vitro* (Roodyn, Suttie & Work, 1962; Simpson, Skinner & Lucas, 1961; Wintersberger, 1965). The inability of ribonuclease to inhibit the incorporation process is considered as a proof for rejecting microsomal contamination as a possible source of the incorporation (McLean *et al.* 1958; Roodyn *et al.* 1961). There are also many indirect lines of evidence showing that the incorporation is not due to contaminating bacteria (Roodyn *et al.* 1961; Roodyn, Freeman & Tata, 1965; Wheeldon, 1966).

However, Decken, Löw & Sandell (1966) re-emphasized the problem of bacterial contamination in the study of incorporation of radioactive amino acid into protein by isolated mitochondria and indicated that the incorporation found by others might be due to bacterial contamination. More recently, however, it has been shown that rat liver mitochondrial preparations that are virtually free from bacteria do incorporate amino acid into

protein (Beattie, Basford & Koritz, 1967; Kroon, Saccone & Botman, 1967; Work, 1968). On the other hand, Sandell, Löw & Decken (1967) confirmed and extended their earlier findings that mitochondrial preparations from rat liver, when free from bacterial contamination, were incapable of incorporating radioactive amino acid into protein; these preparations were, however, fully active in oxidative phosphorylation.

We have examined the reasons for the difference between the results of Sandell *et al.* (1967) and those of the other workers. Our results indicate that the high osmolarity of the incubation medium used by Sandell *et al.* (1967) inhibited protein synthesis by isolated mitochondria. Decreasing the amount of sucrose and omitting tris-hydrochloric acid from this medium to decrease its osmolarity to a level equivalent to that of isotonic sucrose allowed incorporation to proceed at a substantial rate. It has also been shown that the amino acid incorporation into protein by *Escherichia coli* B is only slightly inhibited at an osmolarity that completely stops protein synthesis by isolated mitochondria.

METHODS AND MATERIALS

Isolation of mitochondrial fraction. Hooded rats from Quebec Breeding Farm Inc., St Eustache, P.Q., Canada, were used. For studying amino acid incorporation into protein, the mitochondrial fraction was isolated in 0.3M-

sucrose-2mm-EDTA, pH 7.2, by the method of Roodyn *et al.* (1961). To study oxidative phosphorylation, the mitochondrial fraction was prepared by the method of Schneider & Hogeboom (1950) with the medium of Chance & Hagihara (1962).

Prevention of and assaying for bacterial contamination. To avoid bacterial contamination all equipment used was autoclaved at 120° under steam at 15 lb./in.² for 20 min. The isolation medium as well as the individual solutions of the incubation media (except ADP) were also autoclaved under the same conditions.

Bacterial contamination was checked by plating a sample of the mitochondrial suspension, before and after incubation, on nutrient agar and incubating at 37° for 24 hr.

Incorporation of radioactive amino acid into protein by the mitochondrial preparation. The incubation medium used is described below for each particular experiment. Medium A had the following composition (final concentrations): 0.25 M-sucrose, 50 mM-KCl, 0.67 mM-EDTA, 10 mM-sodium succinate, 20 mM-potassium phosphate, 1 mM-ADP, 5 mM-MgCl₂, 50 mM-tris-HCl buffer, pH 7.5, and 50 µg. of synthetic amino acid mixture/ml. This medium is equivalent to the medium B of Sandell *et al.* (1967). Improved medium A is the same as medium A except that it has 0.1 M-sucrose and no tris-HCl. Medium B (Roodyn *et al.* 1961) had the following composition (final concentrations): 0.1 M-sucrose, 40 mM-KCl, 1.3 mM-EDTA, 10 mM-sodium succinate, 16 mM-potassium phosphate, 4 mM-AMP, 0.5 mM-NAD⁺, 8 mM-MgSO₄ and 50 µg. of synthetic amino acid mixture/ml. Non-radioactive leucine was omitted from the amino acid mixture and L-[1-¹⁴C]leucine was added to a final concentration of 0.6 µCi/ml. There was usually about 3 mg. of mitochondrial protein/ml.

Incubations were carried out with air as the gas phase at 30° with a shaking rate of 80 cycles/min. The reaction was stopped by adding a 0.2 ml. portion of the incubation mixture to 2 ml. of 5% (w/v) trichloroacetic acid containing 0.2 ml. of saturated non-radioactive leucine. The mixture was heated in a water bath at 90° for 15 min. and was then filtered through a nitrocellulose filter (1.2 µ pore size). The protein was washed twice with 5 ml. of 5% (w/v) trichloroacetic acid and finally with 1 ml. of water. The paper was glued on an aluminium planchet and dried and its radioactivity measured with a Nuclear-Chicago gas-flow counter with a counting efficiency of 17%.

RNA synthesis. RNA synthesis was measured in modifications of medium A and medium B by the method of S. Fukamachi & K. B. Freeman (unpublished work). The synthetic amino acid mixture was omitted in both cases and, instead of an adenosine nucleotide, CMP, GMP and AMP were added to a final concentration of 2 mM each. Incubations were carried out at 37° with [³H]UTP at a final concentration of 1 µCi/ml. Samples (0.6 ml.) were added to an ice-cold mixture containing 0.3 ml. of saturated pyrophosphate and 0.2 ml. of non-radioactive UTP (10 mg./ml.) and mixed well. After 10 sec. 3 ml. of ice-cold 5% (w/v) trichloroacetic acid was added. The precipitate was washed with three 3 ml. lots of 5% (w/v) trichloroacetic acid at 0°, dissolved in 0.2 ml. of NCS solvent and transferred into a counting vial with 5 ml. of dioxan scintillation solution, and counted with a Nuclear-Chicago liquid-scintillation counter at 30% efficiency.

Osmometry. The osmolarity of solutions was measured with an osmometer (Advanced Instruments Inc., Newton

Highlands, Mass., U.S.A.). The instrument read-out is expressed as milliosmolarity and these values were converted into the molarity of an equivalent sucrose solution by using a calibration curve of milliosmolarity versus sucrose concentration.

Measurement of oxidative phosphorylation. Succinoxidase activity was measured polarographically as described by Freeman & Haldar (1968) in the media listed in Table 3. The ADP/O ratio was measured by the method of Chance & Williams (1955) and the respiratory control ratio was calculated from the state 3/state 4 rates of oxygen consumption (Lehninger, 1964).

Determination of ATP. The conditions for incubation were same as described above under 'Incorporation of radioactive amino acid into protein by the mitochondrial preparation'. Samples from the incubation mixture were added to HClO₄ to give a final concentration of 0.34 N. After centrifugation, a portion of the supernatant was adjusted to pH 7.4 with 1 N-KOH. After 1 hr. at 0°, the ATP content of the supernatant was measured by following the reduction of NADP⁺ spectrophotometrically at 340 mµ with the combined hexokinase-glucose 6-phosphate dehydrogenase method (Lamprecht & Trautschold, 1965). The reaction mixture contained (final concentrations): 33 mM-glucose, 20 mM-potassium phosphate buffer, pH 7.4, 5 mM-MgSO₄, 33 µg. of hexokinase/ml., 100 µg. of glucose 6-phosphate dehydrogenase/ml. and 67 µg. of NADP⁺/ml. The reaction slowed after 5 min., but could be brought to completion by further additions of 67 µg. of NADP⁺/ml. at 5 and 10 min.

Measurement of amino acid uptake by isolated rat liver mitochondria. The incubations were performed as described above under 'Incorporation of radioactive amino acid into protein by the mitochondrial preparation'. The method of measuring uptake is based on that of Winkler, Bygrave & Lehninger (1968) for the uptake of nucleotides by mitochondria. A 0.1 ml. sample was taken with a pre-cooled ice-cold pipette and was placed on a nitrocellulose filter (0.45 µ pore size) that had been cooled just beforehand by passing 10 ml. of ice-cold 0.3 M-sucrose. The sample was filtered under 6 cm. pressure and was washed with three 1 ml. lots of ice-cold 0.3 M-sucrose. The filter was glued to an aluminium planchet and dried and its radioactivity measured in a gas-flow counter.

Measurement of the formation of aminoacyl-t-RNA in isolated mitochondria.* The incubations were carried out with [1-¹⁴C]leucine as described above. After the incubation, two 0.2 ml. samples were taken. One sample was treated with 2 ml. of ice-cold 5% (w/v) trichloroacetic acid containing 0.2 ml. of a saturated solution of non-radioactive leucine; the mixture was filtered on a nitrocellulose filter at 0° and the precipitate was washed with 10 ml. of ice-cold 5% (w/v) trichloroacetic acid and finally with 2 ml. of ice-cold water. The other 0.2 ml. sample was treated with 2 ml. of 5% (w/v) trichloroacetic acid containing 0.2 ml. of non-radioactive leucine and the mixture was then heated in a water bath at 90° for 15 min.; the mixture was filtered and the precipitate was washed with 5% (w/v) trichloroacetic acid and finally with water. The radioactivity of the samples was measured in a gas-flow counter. The difference in radioactivity between the non-heated and the heated samples was considered to be a measure of the leucyl-t-RNA

* Abbreviation: t-RNA, transfer RNA.

formed because RNA only and not protein would be degraded by heating (Schneider, 1945). In one experiment the incubation mixture was added to 0.1 M-glycine-NaOH buffer, pH 10.3, for 1 hr. at 37° and then treated with ice-cold 5% (w/v) trichloroacetic acid. The precipitate was filtered and washed at 0° as described above and its radioactivity then measured. The difference in radioactivity between the non-heated and alkali-treated samples was also considered to be a measure of the leucyl-t-RNA formed (Moldave, 1963).

Cultivation of *E. coli* B and study of their amino acid incorporation into protein. *E. coli* B was grown in the minimal medium of Davis & Mingioli (1950) and used in mid-exponential phase. Cells were spun down in the cold for 5 min. at 3000g and resuspended in sterilized 0.3 M-sucrose-2 mM-EDTA, pH 7.2, and used immediately. Amino acid incorporation into protein was studied as described under 'Incorporation of radioactive amino acid into protein by the mitochondrial fraction' except that [¹⁴C]leucine (51 µC/µmole) was diluted with non-radioactive leucine to 17 µC/µmole and used at the concentrations described in the particular experiment.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used with bovine serum albumin as standard.

Chemicals. All chemicals used were of reagent grade. Amino acids were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Nucleotides were from C. F. Boehringer G.m.b.H., Mannheim, Germany. Hexokinase (type V), glucose 6-phosphate dehydrogenase (grade II) and tris were products of Sigma Chemical Co., St Louis, Mo., U.S.A. NADP⁺ was obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A. L-[1-¹⁴C]-Leucine was obtained from The Radiochemical Centre, Amersham, Bucks., and had a specific radioactivity of 51 µC/µmole. [5,6-³H]UTP (tetralithium salt) was obtained from Schwarz BioResearch Inc., Orangeburg, N.J., U.S.A., and had a specific radioactivity of 2.0 mc/µmole. Scintillation liquid contained 0.5% (w/v) 2,5-diphenyloxazole, 0.05% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 15.5% (w/v) naphthalene in dioxan. NCS solvent was obtained from Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.

RESULTS

Comparison of the methods of different workers used to study protein synthesis by isolated mitochondria. To determine why Sandell *et al.* (1967) did not observe protein synthesis by isolated mitochondria, we compared their methods with those of other groups who have observed synthesis. The methods of isolation of the mitochondrial fraction did not differ significantly (see Table 9 of Sandell *et al.* 1967), but the incubation medium used by Sandell *et al.* (1967) was different in composition from those used by others. The compositions of some of these media are presented in Table 1. The medium of Sandell *et al.* (1967) (medium I) has the following significant differences from the other media: (a) it has a higher sucrose concentration, 0.25 M, than the 0.1 M-sucrose in the

Table 1. *Compositions of different media used for studying amino acid incorporation by rat liver mitochondrial preparations*

The media described are: I (Sandell *et al.* 1967); II (Roodyn *et al.* 1961); III (Kroon *et al.* 1967).

	Medium I	Medium II	Medium III
Sucrose	0.25 M	0.1 M	—
KCl	50 mM	40 mM	50 mM
EDTA	—	1.3 mM	1 mM
Succinate	10 mM	10 mM	30 mM
Potassium phosphate	20 mM	16 mM	20 mM
AMP	—	4 mM	—
ADP	1 mM	—	2 mM
NAD ⁺	—	0.5 mM	—
MgCl ₂ *	5 mM	8 mM	5 mM
Synthetic amino acids	46 µg./ml.	50 µg./ml.	50 µg./ml.
Tris-HCl buffer	50 mM	—	50 mM
pH	7.5	7.0-7.2	7.4

* MgSO₄ in medium II.

medium of Roodyn *et al.* (1961) (medium II) or no sucrose in the medium of Kroon *et al.* (1967) (medium III); (b) the concentration of adenine nucleotide in medium I is lower than in the other two media; the ratio of adenine nucleotide to Mg²⁺, which might be important for optimum incorporation (Roodyn *et al.* 1961), is also much lower in medium I (1:5) compared with 1:2 in medium II and 1:2.5 in medium III; (c) there is no EDTA in medium I whereas the other two media contain EDTA. Sandell *et al.* (1967) found that addition of EDTA to a medium essentially similar to medium I stimulated incorporation somewhat. Because of these differences the effect of the media on mitochondrial protein synthesis was examined. It should be noted that media I and II are essentially the same as media A and B respectively, which are described in the Methods and Materials section.

Incorporation of [¹⁴C]leucine into protein by isolated rat liver mitochondria suspended in different incubation media. The incorporation of [¹⁴C]leucine into protein by isolated mitochondria in the different media is shown in Fig. 1(a). It is obvious from this result that in medium A (curve A) the incorporation process was severely inhibited compared with that in medium B (curve B). The mitochondrial preparations were checked for bacterial contamination before and after incubation. Although a small number of bacterial cells (less than 200/ml.) could occasionally be detected from a few of the incubated samples, the original preparations never showed any sign of contamination when two 0.1 ml. samples were plated.

The factor or factors in medium A responsible for the inhibition of protein synthesis were then determined. As medium A contained more sucrose

Fig. 1. Incorporation of $[^{14}\text{C}]$ leucine into protein by isolated rat liver mitochondria incubated in different media. Mitochondrial preparations were incubated at 30° with $[^{14}\text{C}]$ leucine ($0.6 \mu\text{C}/\text{ml.}$). The reaction was stopped by adding a 0.2 ml. portion of the incubation mixture to 2 ml. of 5% trichloroacetic acid containing 0.2 ml. of saturated leucine solution. Other details are given in the text. The incubation media were as follows: $\square$, medium A; $\circ$, medium B; $\diamond$, medium A with less sucrose (0.1 M); $\blacksquare$, medium A with more ADP (2 mM); $\blacktriangle$, medium A with less sucrose (0.1 M) but more ADP (2 mM); $\bullet$, medium A with less sucrose (0.1 M) but more ADP (2 mM) and EDTA (1.3 mM); ∇ , medium B with more tris-HCl (50 mM , $\text{pH } 7.5$); $\triangle$, medium A with less sucrose (0.1 M) and no tris-HCl; $\blacklozenge$, medium B with 0.15 M -sucrose; $\blacktriangledown$, medium B to which concentrated solutions of sucrose and tris-HCl, $\text{pH } 7.5$, were added at 10 min. to give final concentrations of 0.25 M (total) and 50 mM respectively.

and tris-hydrochloric acid and less adenine nucleotide and EDTA than medium B, the effects of these differences were examined and are also shown in Fig. 1(a). These results show that decreasing the sucrose concentration in medium A (curve C) improved the incorporation and addition of more tris-hydrochloric acid to medium B (curve G) inhibited the incorporation by isolated mitochondria to a significant extent. Increasing the osmotic pressure in medium B by the addition of tris-hydrochloric acid caused inhibition, whereas lowering the osmotic pressure in medium A by decreasing the sucrose concentration improved the rate of incorporation. These results indicate that the difference in osmotic pressure of the incubation media is possibly the important factor. Addition of more ADP to medium A had no significant effect (curve D), but more ADP when added to medium A with decreased sucrose concentration was slightly inhibitory (curve E). Addition of more EDTA to the latter solution had no significant effect (curve F).

Within the limits of the changes of ADP and EDTA concentrations these factors did not significantly affect incorporation. The effect of omission of tris-hydrochloric acid from medium A in addition to decreasing the sucrose concentration to that of medium B (0.1 M) is shown in Fig. 1(b). It was found, somewhat surprisingly, that this new medium (curve C) was better even than medium B (curve B in Fig. 1b). Medium A therefore was potentially very good for the incorporation of amino acids into protein by isolated mitochondria, but in the hyperosmotic solution the process of incorporation was inhibited. In Fig. 1(b) the inhibitory effects of tris-hydrochloric acid (curve D) and more sucrose (curve E) when added to medium B are also shown. Addition of both tris-hydrochloric acid and more sucrose to medium B to concentrations equivalent to those present in medium A immediately stopped the incorporation process (curve B' in Fig. 1b).

As the main difference between the media seemed

Table 2. *Osmolarity of different media*

Osmolarities were measured with an osmometer as described in the Methods and Materials section and converted into the molarity of an equivalent sucrose solution by using a calibration curve of milliosmolarity versus sucrose concentration.

Medium or solution	Osmolarity of medium (m-osmoles/l.)	Molarity of sucrose equivalent to osmolarity of medium (M)
Medium A	582	0.45
Medium B	296	0.25
Improved medium A	301	0.25
Improved medium A + 0.075 M-sucrose	398	0.32
Improved medium A + 0.15 M-sucrose	495	0.39
Improved medium A + 0.3 M-sucrose	712	0.53
Improved medium A + 25 mM-tris-HCl	340	0.28
Improved medium A + 50 mM-tris-HCl	385	0.31
Improved medium A + 100 mM-tris-HCl	472	0.37
Citrate (20 mM)	67	0.06
β -Hydroxybutyrate (20 mM)	44	0.03
Oxaloacetate (20 mM)	48	0.04
α -Oxoglutarate (20 mM)	62	0.05
Succinate (20 mM)	49	0.04

to be due to the different osmolarity these were accurately determined and the values are shown in Table 2. Also included are values for other solutions whose use is described or discussed below. All values were referred to a standard curve for sucrose solutions. It is obvious from Table 2 that medium A has approximately double the osmolarity of medium B or improved (iso-osmotic) medium A (curve *C* in Fig. 1b).

To show further that the inhibition in medium A was an osmotic effect the following experiment was performed. Amino acid incorporation into protein by isolated mitochondria was studied in the improved medium A. To this medium different concentrations of sucrose and tris-hydrochloric acid were added separately. Fig. 2 (open symbols) shows that increasing the concentration of either sucrose or tris-hydrochloric acid resulted in decreased incorporation of [14 C]leucine into protein of the rat liver mitochondrial preparation. When the molarities of the media containing tris-hydrochloric acid and sucrose are expressed in terms of equivalent sucrose molarities (Table 2) then the curves (solid symbols in Fig. 2) are similar, although the tris-hydrochloric acid caused a slightly greater inhibition. We next examined several other processes to see whether the effect of hyperosmotic solutions on protein synthesis was direct or indirect.

Incorporation of [3 H]UTP into cold-acid-insoluble material. The incorporation of [3 H]UTP into RNA in media A and B is shown in Fig. 3. It is obvious that the incorporation of [3 H]PUT into cold-trichloroacetic acid-insoluble material is not inhibited in medium A. Each point in medium A has, however, a lower value than the corresponding

Molarity of sucrose equivalent to osmolarity of medium

Fig. 2. Effect of increasing concentrations of sucrose and tris-HCl on the incorporation of [14 C]leucine into protein by isolated rat liver mitochondria incubated in improved medium A. ○ and □, Effect of the addition of the compounds when expressed in millimolar concentration; ● and ■, same effects when expressed in equivalent sucrose molarities. ○ and ●, Sucrose; □ and ■, tris-HCl, pH 7.5. The samples were incubated for 45 min. at 30°. The other experimental details are given in Fig. 1.

point in medium B, because the composition of the media affected the precipitation procedure differently.

Effect of hyperosmolarity of the medium on oxidative phosphorylation. Table 3 shows the

Fig. 3. Incorporation of $[^3\text{H}]\text{UTP}$ into cold-acid-insoluble material by isolated rat liver mitochondria suspended in media A and B. Incubations were carried out at 37° with $[^3\text{H}]\text{UTP}$ ($1\mu\text{C}/\text{ml.}$). The reaction was stopped by adding a 0.6 ml. sample to an ice-cold mixture of 0.3 ml. of saturated pyrophosphate and 0.2 ml. of non-radioactive UTP ($10\text{mg.}/\text{ml.}$) and mixing well. After 10 sec., 3 ml. of ice-cold 5% trichloroacetic acid was added and the precipitate was worked up as described in the text. $\square$, Medium A; $\circ$, medium B.

results of experiments on the effect of the osmolarity of the suspending medium on the ADP/O ratio, respiratory control ratio and succinoxidase activity in state 3 respiration of isolated rat liver mitochondria. There was no significant difference in any of these parameters between medium 1 and medium 2, which were approximately equivalent to medium B and medium A respectively. At higher sucrose concentrations (media 3 and 4) the mitochondria became uncoupled, as judged by the decreased respiratory control ratio and the lower succinoxidase activity in the presence of ADP.

As there was no significant difference in the ADP/O ratio of the mitochondria in media essentially the same as media A and B, it was thought worth while to measure the ATP concentrations of the mitochondria suspended in these media. The results of such an experiment are given in Table 4. During the 10 min. incubation period almost all of the added ADP (1mM) was converted into ATP in both the media, but protein synthesis by mitochondria in medium A was virtually completely inhibited.

Uptake of $[^{14}\text{C}]\text{leucine}$ by isolated rat liver mitochondria suspended in media A and B. The results of one such experiment are shown in Fig. 4. Although the results varied somewhat from experiment to experiment, one thing was common to all of them: in both media the uptake of amino acid continued similarly up to about 5 min. and then the amount of the radioactive amino acid decreased in the mitochondria suspended in medium A whereas the radioactivity of mitochondria suspended in medium B went on increasing until about 10 min. The intramitochondrial radioactivity at 10 min. in medium A was about one-third of that in medium B. There was therefore a substantial difference between the concentrations of $[^{14}\text{C}]\text{leucine}$ in the mitochondria suspended in the two different media. The radioactivities measured at zero time were equal to those due to the non-

Table 3. *Effect of osmolarity of the medium on succinoxidase activity*

Media 1, 3 and 4 contained (final concentrations) 16 mM-potassium phosphate buffer, pH 7.4, 40 mM-KCl and 8 mM-MgCl₂, and there was 50 mM-tris-HCl in media 3 and 4. Medium 2 contained (final concentrations) 20 mM-potassium phosphate buffer, pH 7.4, 40 mM-KCl, 5 mM-MgCl₂ and 50 mM-tris-HCl. The concentration of sucrose was 0.1, 0.25, 0.4 and 0.5 M for media 1, 2, 3 and 4 respectively and each medium contained 10 mM-sodium succinate. In each case 0.15 ml. of mitochondria in 0.225 M-mannitol-0.075 M-sucrose-1 mM-EDTA, pH 7.2, was added to 3 ml. of the medium and preincubated at 30° for 3 min. before the addition of ADP.

Medium	Molarity of sucrose equivalent to osmolarity of medium (M)	ADP/O ratio	Respiratory control ratio	Succinoxidase activity in state 3 respiration ($\mu\text{moles of O}_2/3\text{ min.}/\text{mg. of protein}$)
1	0.23	1.68	4.16	65
2	0.42	1.84	3.77	59
3	0.56	1.80	2.57	27
4	0.66	—	1.06	15

Table 4. Concentration of ATP in rat liver mitochondrial preparation after incubation in different media

Mitochondria were incubated as described in the Methods and Materials section. At 30 sec. and 10 min. samples were removed for the determination of ATP, and at 10 min. samples were removed to determine the amino acid incorporation.

Incubation medium	ATP (as % of added ADP)		Specific radioactivity of protein at 10 min. (counts/min./mg. of protein)
	At 30 sec.	At 10 min.	
Medium A	22	92	2
Improved medium A	25	95	62

specific adsorption of [^{14}C]leucine on the filters. The difference in amino acid incorporation into protein by the same mitochondrial preparation (lower part of Fig. 4) suspended in the two media was more marked with virtually no incorporation of [^{14}C]leucine into protein in medium A during the incubation.

Two further points should be noted. First, part of the apparent uptake of [^{14}C]leucine in medium B was due to protein synthesis and with both media part was due to the formation of leucyl-t-RNA. Secondly, only about 0.5% of the added [^{14}C]leucine was taken up by the mitochondria.

Formation of leucyl-t-RNA in rat liver mitochondria suspended in the two media. In the above experiments for determining the amino acid uptake by rat liver mitochondria, the reaction was stopped by immediate filtration at 0° and the mitochondrial samples were washed with ice-cold 0.3M-sucrose. It was possible that some of the radioactive leucine was trapped inside the mitochondria as leucyl-t-RNA that was neither washed out by sucrose nor was accounted for by protein synthesis. As leucyl-t-RNA is stable to ice-cold 5% (w/v) trichloroacetic acid but labile to dilute alkali or hot acid, the difference between the incorporation of radioactivity from [^{14}C]leucine into alkali- or hot-acid-stable material and cold-acid-stable material was taken as a measure of the amount of [^{14}C]leucyl-t-RNA formed. As shown in Table 5 the amount of leucyl-t-RNA formed in medium A was 36–58% of that formed in medium B, whereas the amount of protein synthesis was only 5–10%. The amount of leucyl-t-RNA measured by alkali-labile radioactivity (83.0 counts/min./mg. of protein) was almost the same as that measured by hot-acid-labile radioactivity (96.4 counts/min./mg. of protein).

Synthesis of protein by E. coli B in hyperosmotic media. The inhibition of protein synthesis in

Fig. 4. Uptake of [^{14}C]leucine by isolated rat liver mitochondria incubated in media A and B. Incubations were carried out at 30° with [^{14}C]leucine (0.6 $\mu\text{C}/\text{ml}$). Each sample (0.1 ml.) was filtered and washed at 0° as described in the text. For incorporation experiments, 0.1 ml. samples were precipitated with trichloroacetic acid containing non-radioactive leucine as described in Fig. 1. □, Uptake of [^{14}C]leucine in medium A; ○, uptake of [^{14}C]leucine in medium B; ■, incorporation of [^{14}C]leucine into protein in medium A; ●, incorporation of [^{14}C]leucine into protein in medium B.

isolated mitochondria by hyperosmotic media suggested that one might be able to differentiate between protein synthesis by mitochondria and bacteria if their responses to hyperosmolarity were different. In Fig. 5 the time-course of incorporation of [^{14}C]leucine into protein by *E. coli* suspended in medium A and also in the improved medium A is shown. These results show that for the first 10 min. the rate of amino acid incorporation into protein by *E. coli* suspended in medium A proceeded 50% slower than in the improved medium A. From 10 min. to 30 min. of incubation the rates of incorporation almost paralleled each other; the rate in medium A was only about 15% less. The reason for the initial (10 min.) inhibition of amino acid incorporation by *E. coli* is not clear. It is possible that, on transfer into a medium of high osmolarity, there is a net outflow of solution from the bacterial

Table 5. Incorporation of [^{14}C]leucine into leucyl-t-RNA

Samples were incubated at 37° for 10 min. and then treated as described in the Methods and Materials section.

	Incorporation (counts/min./mg. of protein)		
	Non-heated precipitate	Heated precipitate	Amount due to leucyl-t-RNA
Expt. I			
Improved medium A	141.8	77.8	64.0
Medium A	41.3	4.2	37.1
Expt. II			
Improved medium A	191.0	94.6 (108.0*)	96.4 (83.0†)
Medium A	44.5	9.1	35.4

* The value in parenthesis refers to the radioactivity obtained after treating the sample at 37° for 1 hr. in 0.1 M-glycine-NaOH buffer, pH 10.3, and then precipitating it with ice-cold trichloroacetic acid.

† The value in parenthesis refers to the amount of leucyl-t-RNA as measured by the radioactivity of weak-alkali-labile material.

Fig. 5. Incorporation of [^{14}C]leucine into protein by *E. coli* suspended in medium A and improved medium A. Incubations were carried out at 30° with [^{14}C]leucine (0.1 $\mu\text{C}/\text{ml.}$; specific radioactivity 17 $\mu\text{C}/\mu\text{mole}$) and with 0.9×10^8 cells/ml. The reaction was stopped and the precipitates were worked up as described in Fig. 1 for mitochondrial fractions. ■, Medium A; ○, improved medium A.

Molarity of sucrose equivalent to osmolarity of medium (M)

Fig. 6. Effect of increasing concentrations of sucrose and tris-HCl on the incorporation of [^{14}C]leucine into protein by *E. coli* incubated in the improved medium A. The incubation was at 30° for 10 min. with [^{14}C]leucine (0.13 $\mu\text{C}/\text{ml.}$; specific radioactivity 17 $\mu\text{C}/\mu\text{mole}$) and with 1.33×10^8 cells/ml. The reaction was stopped as described in Fig. 1. ●, Sucrose; ■, tris-HCl, pH 7.5.

cells. In Fig. 6 the effect of increasing concentrations of tris-hydrochloric acid and sucrose on protein synthesis by *E. coli* is shown. In contrast with the results with mitochondria (Fig. 2) sucrose has a smaller effect and tris-hydrochloric acid no effect at all. It is therefore possible to differentiate between the synthesis of protein by mitochondria

and *E. coli* by using hyperosmotic media, especially those including tris-hydrochloric acid.

DISCUSSION

From the results presented here it is clear that the failure of the mitochondrial preparations from

rat liver to incorporate radioactive amino acid into their proteins as observed by Sandell *et al.* (1967) is due, not to the intrinsic incapability of these organelles to do so *in vitro*, but mainly to the medium in which they were incubated. From our results and from the results of other workers (Sandell *et al.* 1967; Work, 1968; Beattie *et al.* 1967; Kroon *et al.* 1967) it is evident that it is certainly possible to prepare a mitochondrial fraction from rat liver virtually free from bacterial contamination provided that one takes proper precautions such as the use of sterilized equipment and solutions. In our experience the isolation medium, which contains sucrose, is one of the most important sources of bacterial contamination. It was sterilized before each experiment and left uncovered as little as possible. We also confirm that bacteria-free mitochondrial preparations from rat liver can synthesize protein if incubated in a suitable medium (Work, 1968; Beattie *et al.* 1967; Kroon *et al.* 1967).

The osmolarity of the medium A is approximately equivalent to that of 0.45M-sucrose solution (Table 2), which has nearly double the concentration of iso-osmotic sucrose (0.25M). A decrease in the concentration or complete removal of the solutes, such as sucrose and tris-hydrochloric acid, that were responsible for the high osmolarity of the medium, improved the medium to a remarkable extent with respect to its ability to support amino acid incorporation into protein by isolated mitochondria (Fig. 1b). It is noteworthy that the osmolarity of the resulting improved medium is equivalent to 0.25M-sucrose (Table 2), which is considered to be iso-osmotic.

The inhibitory action of the medium of Sandell *et al.* (1967) is considered to be an osmotic effect because the extent of inhibition obtained by adding more sucrose or tris-hydrochloric acid to an iso-osmotic medium depended primarily on the resulting osmolarity, and not on the nature of the added solute molecule. It is not to be expected, however, that the inhibition will be completely comparable with all types of solutes if they are added to equivalent osmolar concentrations, as the resultant inhibition will depend to a great extent on the permeability of mitochondria to the molecule and also on special inhibitory property of the molecule, if any. Melamed & Recknagel (1959), Pfaff (1967a) and many others have found that sucrose enters only a part of the intramitochondrial water space whereas the rest responds as an osmometer to changes in sucrose concentration. The latter is probably the inner mitochondrial compartment. Azzone & Bergh (1967) found that tris ion behaves somewhat like sucrose in respect to uptake by mitochondria.

Roodyn (1965) reported that some, but not all, of

the tricarboxylic acid-cycle intermediates at low concentrations stimulated, but at higher concentrations inhibited, protein synthesis by isolated mitochondria. The osmolarities of some of these compounds at 20mM concentration (the maximum concentration used by Roodyn, 1965) were determined (Table 2). The inhibition or the lack of inhibition by these compounds at the said concentrations can be at least partially explained on the basis of the osmolarity of their solutions. Thus malate, succinate, β -hydroxybutyrate and hexanoate did not inhibit, but oxaloacetate, *cis*-aconitate and citrate did. From the results of Chappell & Haarhoff (1967) and Meiger, De Haan & Tager (1967) it would be expected that the mitochondria would be permeable to the former group but not the latter. The latter compounds only would increase the osmolarity of the medium with respect to the mitochondrial osmoticity and thus inhibit protein synthesis.

Our results indicate no marked difference between medium A and medium B with respect to their effect on oxidative phosphorylation. At osmolarity equivalent to 0.56M-sucrose or above (media 3 and 4 in Table 3) there was an uncoupling of the respiration of rat liver mitochondria and a decreased succinoxidase activity. Protein synthesis by mitochondrial preparations was inhibited at a lower osmolarity of the incubation medium than that required to inhibit oxidative phosphorylation.

Although the incorporation of radioactive amino acid into protein was virtually completely inhibited in medium A, the intramitochondrial concentration of free [14 C]leucine was about one-third of that when the mitochondrial fraction was incubated in medium B for 10min. (Fig. 4), when allowance is made for the protein synthesized and leucyl-t-RNA. This difference in the intramitochondrial concentration of [14 C]leucine is not sufficient to explain the inhibition of protein synthesis in medium A. In addition to this, the incorporation of [14 C]leucine into cold-acid-insoluble material that was alkali-labile was even less inhibited (Table 5). It seems likely that the latter incorporation is into leucyl-t-RNA of the mitochondria. If this is correct, then it appears that it is the part of the protein-synthetic mechanism occurring on mitochondrial ribosomes that is being affected by high osmolarity. The decrease in the labelling of t-RNA might be a secondary effect because the original non-radioactive aminoacyl-t-RNA is not being utilized. Although the presence of leucyl-t-RNA and leucyl-t-RNA synthetase has been shown in extracts of rat liver mitochondria (Buck & Nass, 1968) and mitochondria from other tissues (Suyama & Eyer, 1967; Barnett, Brown & Epler, 1967), this is the first demonstration of its formation in whole mitochondria.

A further experiment supporting the idea that the inhibition of protein synthesis is at a later stage of the process is the almost complete cessation of protein synthesis when sucrose and tris-hydrochloric acid were added to mitochondria after 10 min. incubation in medium B (curve *B'* in Fig. 1b). It would be expected that the concentrations of [^{14}C]leucine and [^{14}C]leucyl-t-RNA would be sufficient for much further synthesis unless the process was blocked at the level of the mitochondrial ribosomes.

These arguments do not necessarily mean that the difference in the intramitochondrial concentration of amino acids has nothing to do with the inhibition of protein synthesis in medium A, but the main block in the amino acid incorporation into protein seems to be located at a much later step of the synthesis as described above. The picture of the uptake of amino acids by isolated mitochondria is not clear at present. With the methodology we used only 0.5% of the added [^{14}C]leucine was trapped inside the mitochondria. Garfinkel (1963) investigated the penetration of glycine and some other amino acids into isolated mitochondria, but it was not clear whether the transport was active or passive. Wheeldon & Lehninger (1966) indicated that the transport is an active process because the uptake is stimulated by Mg^{2+} and ATP, but no data were given.

Slater & Cleland (1953) and Chappell & Greville (1963) have reviewed much of the early work on the morphology and activity of mitochondria isolated in 0.88 M-sucrose or iso-osmotic sucrose. Generally, mitochondria isolated in media with high sucrose concentration have the rod-like appearance of mitochondria *in situ*, but have much lower respiratory activity. Slater & Cleland (1953) found that an increase in sucrose concentration progressively inhibited the α -oxoglutarate oxidase system of rat heart mitochondria, but the P/O ratio was unaffected. In contrast, Pfaff (1967b) states in a discussion of a symposium that above 0.4 M-sucrose the respiration of rat liver mitochondria decreases and what is left is uncoupled. Perhaps of greatest bearing on our studies is the result obtained by Whittaker (1966). He found that rat liver mitochondria isolated in 0.44 M-sucrose (an osmolarity equal to medium A) were elongated and had a dense matrix with few and narrow cristae when fixed with osmium tetroxide and negatively stained. In contrast, mitochondria isolated in 0.25 M-sucrose (i.e. an osmolarity equivalent to medium B or improved medium A) were round and swollen. It is possible that in the high osmolarity the contracted state of the mitochondria in some way inhibits protein synthesis.

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