Biochem. J. (1967) **104**, 575

A Critical Study of Amino Acid Incorporation into Protein by Isolated Liver Mitochondria from Adult Rats

BY SYLVIA SANDELL, H. LÖW AND ALEXANDRA VON DER DECKEN
The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Stockholm, Sweden

(*Received* 5 *December* 1966)

1. Mitochondria were isolated from rat liver in a way that kept bacterial contamination at a minimum. 2. The activity of oxidative phosphorylation was unchanged under these conditions, whereas the ability of the preparations to incorporate amino acids into protein was insignificant, though it could be enhanced somewhat by the presence of EDTA. This enhancement was sensitive to ribonuclease. 3. The active time of incorporation did not exceed 15min. at 30°. 4. Microsomal contamination, as measured by glucose 6-phosphatase activity, was about 5%. 5. The ability of isolated bacteria to incorporate amino acids into protein was greatly enhanced by the addition of mitochondria or heat-inactivated mitochondria. 6. A correlation was found between the growth rate of bacteria and the amino acid-incorporating activity. 7. Amino acid incorporation by combined mitochondrial–bacterial systems was inhibited by 2,4-dinitrophenol. 8. The results confirm and extend the earlier findings made in our Laboratory that isolated liver mitochondria, when free from contaminating bacteria and obtained from adult rats, are not able to catalyse the incorporation of amino acids into protein at a measurable rate. 9. The results are discussed with special emphasis on the validity of these findings.

It has recently been demonstrated that, although isolated rat-liver mitochondria are not able to synthesize soluble proteins, they are active in incorporating labelled amino acids into protein (Roodyn, 1962; Roodyn, Suttie & Work, 1962; Truman, 1964). It has been suggested that the amino acids are presumably incorporated into structural membrane protein. However, the conditions for amino acid incorporation differed from those for microsomes, particularly with regard to ribonuclease-sensitivity (McLean, Cohn, Brandt & Simpson, 1958).

Results presented earlier (Decken, Löw & Sandell, 1966) indicated that the incorporation of amino acids into protein found by others might be due to bacterial contamination. This possibility has been considered by several workers, but has been rejected (Roodyn, Reis & Work, 1961; Kalf, 1963; Roodyn, Freeman & Tata, 1965). In view of the differences it was decided to educe further evidence on the significance of bacterial contamination for mitochondrial amino acid incorporation.

A preliminary report of this work has appeared (Sandell, Löw & Decken, 1966).

EXPERIMENTAL

Chemicals. ADP, ribonuclease, tris hydrochloride (crystallized), tris base (recrystallized) and oligomycin were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Non-radioactive amino acids were obtained from California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.). A synthetic mixture was prepared as described by Roodyn *et al.* (1961). Puromycin was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and 2,4-dinitrophenol was from Merck, Sharp and Dohme Inc. (Rahway, N.J., U.S.A.). Bacto nutrient broth, Bacto nutrient agar and Bacto yeast extract were obtained from Difco Laboratories Inc. (Detroit, Mich., U.S.A.). Blood-agar plates were obtained from the Karolinska Hospital (Stockholm, Sweden). [1-^{14}C]Glycine (1·5mc/m-mole), DL-[1-^{14}C]valine (46mc/m-mole), DL-phenyl[1-^{14}C]alanine (47·5mc/m-mole), DL-[2-^{14}C]tyrosine (9·6mc/m-mole) and L-[^{14}C]proline (12·6mc/m-mole) were obtained from The Radiochemical Centre (Amersham, Bucks.). L-[^{14}C]Lysine (12·5mc/m-mole), DL-[3,4-^{14}C$_2$]glutamic acid (10mc/m-mole) and DL-[1-^{14}C]leucine (20mc/m-mole) were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.).

Preparation of mitochondria. To avoid bacterial contamination of the mitochondrial preparations, sterilization of materials and chemicals before use was as follows. Glassware, instruments and centrifuging tubes were

sterilized for 45 min. at 1·4 kg./cm.^{2}, preparative media for 20 min. at 1·4 kg./cm.^{2}. The cofactors and substrates of the incubation mixture such as ADP and succinate were passed through a membrane filter (pore size 0·25 μ). The total loss of material by filtration was about 0·5 ml. The concentration was not significantly altered as tested by determination of ADP present in the filtrate. The preparation procedures were carried out at 0–4°.

Non-starved Sprague–Dawley male or female rats (120–150 g.) were used. The rats were killed by a blow on the head, decapitated and bled, and the liver of each was removed with scissors and forceps and placed in 0·25 M-sucrose. The liver was washed with four changes of sucrose, minced, passed through a garlic press and homogenized by hand in a Potter–Elvehjem-type Teflon–glass homogenizer. Homogenization was carried out in a volume of sucrose about 10 times that of the weight of the liver. During the investigation it was found that mitochondria essentially free from bacteria could be obtained by homogenization with a motor-driven homogenizer. Treatment of the liver with the garlic press could thus be omitted and, therewith, the preparation of the mitochondria followed closely the procedure described by Ernster & Löw (1955). In test experiments it was found that omission of the garlic press did not influence the results. The homogenate was centrifuged for 10 min. at 500g in a Servall refrigerated centrifuge (type RC–2). The supernatant was centrifuged for 20 min. at 5000g. The mitochondrial pellet was washed twice by suspension and centrifugation. The final pellet obtained was suspended in 0·25 M-sucrose to give a protein content of 20 mg./ml. The time elapsing between the killing of the rat and the start of the incubation was not more than 3 hr.

When heat-inactivated mitochondria were used the suspension was kept in a water bath at 60° for 5 min. and then cooled in an ice bath. As a control of the inactivation of mitochondrial functions respiration was measured (Chance & Williams, 1955).

Method of incubation. Two media were used in the amino acid-incorporation experiments. Medium A contained sucrose (0·25 M), tris–HCl buffer, pH 7·5 (35 mM), KCl (80 mM), $MgCl_2$ (10 mM) and the amino acid mixture (46 μg./ml.) containing all amino acids except the labelled one (Roodyn *et al.* 1961). Medium B contained sucrose (0·25 M), tris–HCl buffer, pH 7·5 (50 mM), KCl (50 mM), $MgCl_2$ (5 mM), potassium phosphate buffer, pH 7·5 (20 mM), sodium succinate (10 mM), ADP (1 mM) and the amino acid mixture (46 μg./ml.). If not otherwise indicated, incubation was carried out in tubes (diam. 2 cm.) covered with aluminium foil. The mitochondria, corresponding to 5 mg. of protein/ml., were incubated in medium A or medium B together with the labelled amino acid (0·25 μC of the L-form/ml.). The final volume was 2 ml. Incubation was at 30° under vigorous shaking of 80 cyc./min. to secure sufficient oxygen supply. Bacterial content, as determined after each incubation by plating a sample of the incubation mixture on nutrient agar or on blood–agar, was below 10 cells/ml.

Isolation and cultivation of bacteria. A sample of a mitochondrial suspension isolated under non-sterile conditions was diluted with sterile cultivation medium containing 8 g. of nutrient broth and 3 g. of yeast extract/l. of deionized water. The suspension was plated on nutrient agar. The plates were kept overnight at 37°. Identification showed the presence of Gram-negative bacteria and of *Escherichia coli*. *E. coli* were isolated, transferred to 2 ml. of medium A and incubated with vigorous shaking for 18 hr. in a water bath at 37°. The bacterial suspension was then transferred to a 300 ml. Erlenmeyer flask containing 30 ml. of medium A. The flasks were incubated under shaking for 3 hr. at 37°. An appropriate counting of the bacteria was carried out in a counting chamber of the Bürker type (0·0025 and 0·04 mm.^{2}). A calculated number of these bacteria was added directly to the incubation tubes containing the medium together with the radioactive amino acids. When *E. coli* of a stationary phase were used a calculated number of bacterial cells was added to a 100–1000-fold excess of ice-cold M-KCl and kept in ice bath for 1 hr. This suspension was then added to the incubation tubes containing the medium and the labelled amino acids. In these experiments the final concentration of KCl was 0·15 M.

An exact determination of the bacterial content was made as a routine before and after incubation by plating a sample on nutrient agar. These data were used when bacterial cell counts are indicated in the paper.

Extraction of proteins. Two procedures were used.

(*a*) The incorporation of radioactive amino acids was stopped by the addition of 1·5 ml. of N-$HClO_4$. A saturated solution of the corresponding non-labelled amino acid (0·5 ml.) was added. The precipitate obtained after centrifugation was resuspended in 0·5 N-$HClO_4$, centrifuged and dissolved in 1 ml. of N-NaOH. After digestion at room temperature for 30 min., the proteins were precipitated by the addition of 2 ml. of N-$HClO_4$ and extracted for 10 min. at 70°. The precipitate was then washed once with ethanol, once with ethanol–ether–chloroform (2:2:1, by vol.) and twice with ether.

(*b*) After incubation the tubes were transferred to an ice bath and 100 μl. of the incubation mixture was pipetted on to each of three filter-paper disks (Munktell, no. 3) of diam. 22 mm. The extraction of the proteins followed the method described by Mans & Novelli (1961), with the modification that the disks were collected by decanting the solutions. In addition, a further extraction with ethanol–ether–chloroform (2:2:1, by vol.) after the extraction with ethanol was included.

Measurement of radioactivity. Two types of apparatus were used.

(*a*) Thin-window gas-flow counter (Nuclear–Chicago Corp.). The ^{14}C-labelled proteins extracted in the centrifuging tubes were dissolved in formic acid, transferred to 7 cm.^{2} aluminium disks and dried under an infrared lamp. The samples were counted at infinite thinness. The amount of protein on the planchet was determined by weight. The radioactivity was expressed as counts/min./mg. of protein. When 0·01 μC of a ^{14}C-labelled amino acid was counted under similar conditions at infinite thinness 4000 counts/min. were obtained, indicating a counting efficiency of 18%.

(*b*) Liquid-scintillation counter (Packard Tri-Carb model 314 EX). The filter-paper disks were counted at 40% efficiency by adding 10 ml. of a scintillation liquid containing 2,5-diphenyloxazole (0·5%) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0·015%) in toluene.

Determination of glucose 6-phosphatase activity. The enzyme activity was determined by a method that followed closely that of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The assay was performed on sufficient mitochondrial suspension to give 0·5–2 mg. of protein. The incubation mixture contained, in a final volume of 0·45 ml.,

glucose 6-phosphate (40mM), EDTA (1mM), citrate buffer (7mM), sucrose (0·15M) and the mitochondrial suspension. The pH of the solutions added was 6·5 (by the pH-meter). Two controls were used, one in the absence of glucose 6-phosphate and one in the absence of the mitochondrial suspension. After 10min. at 37° 1ml. of 10% (w/v) trichloroacetic acid was added. The suspension was centrifuged and the free P_i of the soluble fraction assayed by the method of King, Abul-Fadl & Walker (1951). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Oxidative phosphorylation. The P/O ratios were calculated, from the respiratory 'stairways' induced by ADP, by the method of Chance & Williams (1955). The details are as follows. The reaction mixture contained, in a final volume of 3ml., KCl (50mM), sodium phosphate buffer, pH7·5 (8mM), $MgCl_2$ (4mM), sodium glutamate (7mM) or sodium succinate (7mM) and mitochondria (6mg. of protein). After measurement of the O_2 uptake (state 4), 5μl. of ADP (0·5μmole) was added and the increase in O_2 uptake (state 3) was recorded. When all the ADP had been used up a further 5μl. was added. This was repeated until significant data on the P/O ratio were obtained. The respiratory control (state 3/state 4 ratio) for succinate or glutamate as substrate was calculated.

Removal of non-peptide-bound amino acids. The three methods described in detail by Suttie (1962) were used, namely treatment with performic acid, ninhydrin and thioglycollic acid.

Determination of protein. This was carried out by the method of Gornall, Bardawill & David (1949).

RESULTS

Characteristics of the mitochondrial preparations. The ability of rat-liver mitochondria to incorporate various amino acids into protein was measured. Two media were used. Medium A contained various ions and an amino acid mixture, whereas medium B contained in addition succinate, P_i and ADP.

As shown in Table 1, when the mitochondria were incubated in medium A, there was no significant incorporation of [^{14}C]glycine, [^{14}C]valine, [^{14}C]-leucine, [^{14}C]lysine and [^{14}C]phenylalanine into protein. On the other hand, [^{14}C]tyrosine, [^{14}C]-proline and [^{14}C]glutamic acid were incorporated into protein at a rate of 2–5counts/min./mg. of protein. This radioactivity could have been due to a binding of the amino acids to the proteins by a non-peptide bond; but, when the proteins were treated to remove the non-peptide-bound amino acids the radioactivity was not significantly altered (see the Experimental section and Suttie, 1962).

When mitochondria were incubated in medium B that had been supplemented with EDTA (1mM), it seemed at first that the incorporation of [^{14}C]-leucine could be increased. As shown in Table 2 about 7–10counts/min./mg. of protein were incorporated. The system was partially sensitive to ribonuclease. Of the other inhibitors tested puromycin had an effect similar to that obtained in microsomal amino acid-incorporating systems (Yarmolinsky & De La Haba, 1959). In the presence

Table 1. *Incubation with various* ^{14}C*-labelled amino acids of mitochondrial preparations isolated in* 0·25M*-sucrose*

Mitochondria (5mg. of protein/ml.) were incubated in a medium containing sucrose (0·25M), tris–HCl buffer, pH7·5 (35mM), KCl (80mM), $MgCl_2$ (10mM) and the L-form of the labelled amino acid (0·25μC/ml.) indicated. With [^{14}C]leucine an amino acid mixture (46μg./ml.) containing all amino acids except the labelled one was also added. Incubation was for 2hr. at 30° with vigorous shaking in a final volume of 2ml. After incubation the bacterial content was determined to be below 10 cells/ml. The proteins were precipitated with $HClO_4$ and extracted, and the radioactivity was determined in a thin-window gas-flow counter. The P/O ratios varied from 0·88 to 1·61 with succinate and from 1·28 to 2·85 with glutamate as substrate. The respiratory control was between 2·5 and 3·2 with succinate and between 2·8 and 4·0 with glutamate as substrate. Ranges of the radioactivity values obtained are given in parentheses.

^{14}C-labelled amino acid added	Incorporation (counts/min./mg. of protein)	No. of expts.
Glycine	0·3 (0–0·5)	2
Valine	0	2
Leucine	1·1 (0–2·5)	18
Lysine	0	2
Phenylalanine	0	6
Tyrosine	2·2 (0–5·8)	6
Proline	3·5 (0·8–5·4)	9
Glutamic acid	5·0 (2·8–10·6)	4

Table 2. *Incubation in medium B containing EDTA* (1mM) *of mitochondrial preparations, isolated in* 0·25M*-sucrose*

Mitochondria (5mg. of protein/ml.) were incubated with L-[^{14}C]leucine (0·25μC/ml.) in medium B supplemented with EDTA (1mM). Incubation was for 2hr. at 30° with vigorous shaking in a final volume of 2ml. Inhibitors were added as indicated to the control system. The bacterial content was below 10 cells/ml. After incubation the proteins were precipitated with $HClO_4$ and extracted, and the radioactivity was measured in a thin-window gas-flow counter.

Expt. no.	Addition to system	Incorporation (counts/min./mg. of protein)
1	None (control)	10·2
	Ribonuclease (0·1mg./ml.)	4·1
	2,4-Dinitrophenol (0·1mM)	0·6
2	None (control)	7·0
	Ribonuclease (0·1mg./ml.)	3·1
	Puromycin (1mM)	0
	KCN (1mM)	0

of 2,4-dinitrophenol or cyanide the incorporation was abolished completely.

Fig. 1 shows that under these conditions the preparations were active for only a short time. In several experiments the total period of incubation was prolonged to 1 or 2hr. at 30°, but maximal incorporation was reached usually after 7min. and in a few experiments after 15min. These results are in close agreement with those obtained by Truman & Korner (1962). A low but significant contamination of the mitochondrial preparations by microsomes was expected. To decrease or to render inactive the apparent microsomal contamination, the mitochondria were isolated in the presence of EDTA. As a measure of microsomal content the activity of the microsomal enzyme glucose 6-phosphatase was determined after isolation of the mitochondria in the absence or the presence of EDTA. The results are summarized in Table 3. The glucose 6-phosphatase activity increased when the mitochondrial fractions were prepared in the presence of EDTA. Glucose 6-phosphatase activity measured under similar conditions in microsomes showed an activity of 6·45 μmoles of P_i released/mg. of protein/10min. at 35° (Decken & Campbell, 1964). From these results the contamination by microsomal membranes of the mitochondrial preparations was calculated to about 5%.

The presence of EDTA did not make it possible to prepare mitochondria completely free of microsomes. In the hope that EDTA would dissociate the ribosomes EDTA was included in the medium during isolation of the mitochondria, but was omitted usually during incubation. The ability of these preparations to incorporate [^{14}C]leucine into protein is shown in Table 4. The incorporating activity of the different preparations varies. In some experiments a significant inhibition by ribonuclease was obtained. Three concentrations of EDTA were used. The incorporating activity of the systems was decreased when EDTA was present in the preparative medium, although the concentration of EDTA used seemed not of great importance in this connexion. However, it was observed that the mitochondria had the tendency to aggregate at a concentration of 10mM-EDTA and higher. Therefore 2mM-EDTA was used preferentially. The incorporation of [^{14}C]tyrosine, [^{14}C]proline and [^{14}C]glutamic acid was measured under similar conditions. The results, summarized in Table 5, show an incorporation of 0–1·5counts/min./mg. of protein as compared with the somewhat higher values obtained in mitochondrial preparations isolated in the absence of EDTA (Table 1).

Characteristics of amino acid incorporation into protein by bacteria. As has been shown earlier (Decken *et al.* 1966), rat-liver mitochondria when isolated under non-sterile conditions contain a certain number of micro-organisms. One of the

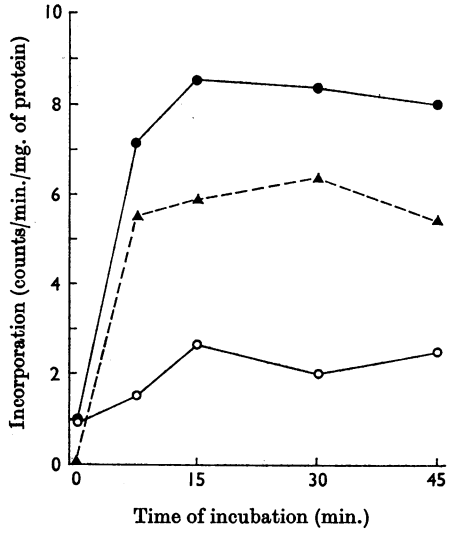

Fig. 1. Mitochondria (5mg. of protein/ml.) were incubated as described in Table 2. Incubation was at 30° for the time indicated. The bacterial content was below 10cells/ml. Precipitation of proteins and radioactivity measurements were as in Table 2. As a control, heat-inactivated mitochondria (5mg. of protein/ml.) were incubated under similar conditions. ●, Active mitochondria; ○, heat-inactivated mitochondria; ▲, values after subtraction of those for heat-inactivated mitochondria.

Table 3. *Activity of glucose 6-phosphatase in mitochondrial preparations*

Each incubation tube contained, in a final volume of 0·45ml., glucose 6-phosphate (40mM), EDTA (1mM), citrate buffer, pH6·5 (7mM), sucrose (0·15M) and the mitochondrial suspension (containing 0·5–2mg. of protein). The tubes were run in duplicates with two concentrations of the mitochondrial preparations. Two controls were used, one in the absence of glucose 6-phosphate and one in the absence of mitochondrial suspension. After incubation in air at 37° the proteins were precipitated in 5% (w/v) trichloroacetic acid. The amount of P_i in solution was determined. The specific activity of glucose 6-phosphatase is expressed as μmoles of P_i obtained/10min./mg. of protein at 37°.

Mitochondria prepared in	Specific activity: Untreated	Specific activity: Treated for 2hr. at 30°
0·25M-Sucrose	0·30	0·17
0·25M-Sucrose–2mM-EDTA	0·97	0·31
0·25M-Sucrose–10mM-EDTA	0·85	0·39

Table 4. *Incubation of mitochondrial preparations isolated in* 0·25 M-*sucrose–EDTA*

Mitochondria (5 mg. of protein/ml.), isolated in 0·25 M-sucrose containing the EDTA concentrations indicated, were incubated in medium B with L-[^{14}C]leucine (0·25 μc/ml.). Inhibitors were added as indicated to the control system. Incubation was for 2 hr. at 30° with vigorous shaking in a final volume of 2 ml. The bacterial content was below 10 cells/ml. After incubation the proteins were precipitated with $HClO_4$ and extracted, and the radioactivity was measured in a thin-window gas-flow counter. The P/O ratios varied from 1·35 to 1·8 and respiratory control was between 2·8 and 3·3 with succinate as substrate.

Expt. no.	Concn. of EDTA during isolation of mitochondria (mM)	Addition to system	Incorporation (counts/min./mg. of protein)
1	15	None (control)	2·8
		Ribonuclease (0·1 mg./ml.)	1·2
		KCN (1 mM)	0·5
		2,4-Dinitrophenol (0·1 mM)	0·1
		Oligomycin (1 μg./ml.)	1·4
2	15	None (control)	1·5
		Ribonuclease (0·1 mg./ml.)	0·1
		2,4-Dinitrophenol (0·1 mM)	0·6
3	10	None (control)	3·4
		Ribonuclease (0·1 mg./ml.)	3·2
4	10	None (control)	1·1
		Ribonuclease (0·1 mg./ml.)	1·0
5	10	None (control)	0
		Ribonuclease (0·1 mg./ml.)	0
6	2	None (control)	0
		Ribonuclease (0·1 mg./ml.)	5
7	2	None (control)	0
		Ribonuclease (0·1 mg./ml.)	0

Table 5. *Incubation with various* ^{14}C-*labelled amino acids of mitochondrial preparations isolated in* 0·25 M-*sucrose–*10 mM-*EDTA*

Mitochondria (5 mg. of protein/ml.), isolated in 0·25 M-sucrose containing EDTA (10 mM), were incubated in medium B with the L-amino acid (0·25 μc/ml.) indicated. Incubation was for 2 hr. at 30° with vigorous shaking in a final volume of 2 ml. The bacterial content was below 10 cells/ml. After incubation the proteins were precipitated with $HClO_4$ and extracted, and the radioactivity was measured in a thin-window gas-flow counter. Ranges of the values obtained are given in parentheses.

^{14}C-labelled amino acid	Incorporation (counts/min./mg. of protein)	No. of expts.
Leucine	1·1 (0–3·4)	9
Tyrosine	0	4
Proline	1·2 (0·6–1·5)	4
Glutamic acid	0·7 (0·4–1·1)	6

strains of the contaminating bacteria has been isolated and characterized as *E. coli*. These bacteria have been used to study the incorporation of amino acids into protein in combined mitochondrial–bacterial systems.

As shown in Table 6, the bacteria incorporated [^{14}C]leucine into protein when incubated in medium A. Table 6 also shows that mitochondria alone, when incubated in parallel experiments, did not incorporate [^{14}C]leucine into protein at a significant rate. However, when the mitochondria were added to the bacteria the combined system showed a capacity to incorporate the labelled amino acid into protein far exceeding that of the bacterial system alone. It is apparent from these data that there was no direct correlation between the number of bacteria added and the incorporation obtained. It is known (Lamborg & Zamecnik, 1960) and was also confirmed in the present investigation that *E. coli* have a higher incorporating activity during exponential growth than during the stationary phase. As shown in Table 6 the stimulation of the amino acid incorporation into protein of the combined mitochondrial–bacterial system varied between 1·5- and 25-fold. This was probably due to the physiological status of the bacteria before being added to the system.

To obtain better-controlled experimental conditions bacteria were added to M-potassium chloride and kept in an ice bath for 1 hr. After such treatment all bacteria were in the stationary phase. They were diluted in medium A to give a final concentration of 0·15 M-potassium chloride and incubated with labelled amino acids in the absence or presence

Table 6. *Effect of added mitochondria on the ability of* E. coli *to incorporate* L-[^{14}C]*leucine into protein*

E. coli were isolated from a mitochondrial suspension that had been prepared under non-sterile conditions. The indicated amount of bacterial cells was incubated in medium A containing L-[^{14}C]leucine (0·25 μc/ml.) in the absence or presence of mitochondria (5 mg. of protein/ml.). In control systems mitochondria were incubated under similar conditions but without the addition of bacterial cells. The number of bacterial cells present was below 10 cells/5 mg. of mitochondrial protein. Incubation was for 2 hr. at 30° with vigorous shaking in a final volume of 2 ml. After incubation the bacterial systems were supplemented with mitochondria (5 mg. of protein/ml.). The proteins were precipitated with $HClO_4$ and extracted, and the radioactivity was measured in a thin-window gas-flow counter. The P/O ratios varied from 0·74 to 1·7 with succinate and from 2·2 to 2·4 with glutamate as substrate. The respiratory control was between 2·4 and 3·6 with succinate and between 3·2 and 5·0 with glutamate as substrate.

			Incorporation (counts/min./mg. of protein)		
Expt. no.	No. of bacterial cells added	Assay system ...	Bacteria	Bacteria + mitochondria	Mitochondria (control)
1	10^5		181	221	1·0
2	10^6		120	164	2·3
3	5×10^3		18	38	1·7
4	2×10^5		12	27	0
5	10^7		1049	2640	0
6	5×10^6		60	1435	1·3

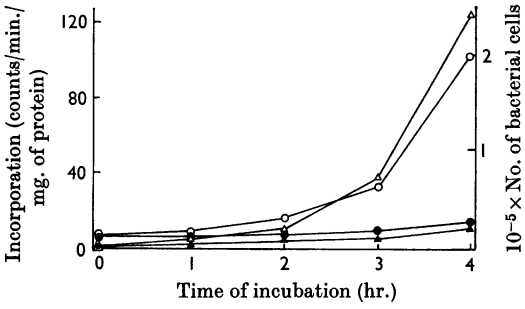

Fig. 2. Growth and amino acid-incorporating capacity of bacteria in the absence and presence of mitochondria. ▲, Radioactivity (counts/min./mg. of protein) after incubation of bacteria; △, radioactivity (counts/min./mg. of protein) after incubation of bacteria and mitochondria; ●, number of cells after incubation of bacteria; ○, number of cells after incubation of bacteria and mitochondria. Before incubation the bacteria had been transferred to the stationary phase by treatment with ice-cold M-KCl. The cells were incubated in medium A containing L-[^{14}C]glutamic acid (0·25 μc/ml.) in the absence or presence of mitochondria (5 mg. of protein/ml.). Incubation was for the times indicated at 30° with vigorous shaking in a final volume of 2 ml. In control systems mitochondria were incubated under similar conditions for 4 hr. A radioactivity of 2·8 counts/min./mg. of protein was obtained. After incubation the bacterial systems were supplemented with mitochondria (5 mg. of protein/ml.). The proteins were precipitated with $HClO_4$ and extracted, and the radioactivity was measured in a thin-window gas-flow counter.

of mitochondria. As shown in Fig 2, the incorporation of ^{14}C-labelled amino acid into protein increased when bacteria and mitochondria were incubated together. The same applies to the division of bacterial cells.

The above experiments suggested that mitochondria had an effect on the division and protein-synthetic activity of bacteria. To study this phenomenon further a comparison was made of the effect of adding heat-inactivated mitochondria to bacterial suspensions. The heat-inactivated mitochondria were not capable of oxidative phosphorylation. The results are presented in Table 7. Both the active mitochondria and the heat-inactivated mitochondria had a stimulatory effect on the bacterial growth. Further, the incorporation of the labelled amino acids into protein was enhanced to about the same extent by the active mitochondria and by the heat-inactivated mitochondria.

Effect of 2,4-dinitrophenol on mitochondrial systems contaminated with bacteria. 2,4-Dinitrophenol is a good uncoupler of oxidative phosphorylation. Its effect on the incorporation of labelled amino acids into protein by mitochondrial preparations has been studied extensively (for references see Table 8). In addition, it has been shown by Simon, Van Praag & Aronson (1966) that net synthesis of RNA in bacteria is inhibited by 2,4-dinitrophenol. These results led us to investigate the effect of 2,4-dinitrophenol on the labelling of proteins in mitochondrial systems contaminated with bacteria in a manner similar to that described by Simon *et al.* (1966) for bacteria. Fig. 3 shows the labelling of proteins at various periods of incubation. At the point indicated by the arrow, 2,4-dinitrophenol was added. A marked decrease in the labelling of proteins was observed in the 2,4-dinitrophenol-treated preparations. The effect of 2,4-

Table 7. *Effect of active and heat-inactivated mitochondria on the growth rate and amino acid-incorporating activity of* E. coli

E. coli were isolated from a mitochondrial suspension that had been prepared under non-sterile conditions. The bacterial cells were transferred to the stationary phase by treatment with ice-cold M-KCl. The cells were incubated in medium A containing L-[^{14}C]glutamic acid (0·25 μc/ml.; Expt. 1) or L-[^{14}C]leucine (0·25 μc/ml.; Expt. 2). Mitochondria (5mg. of protein/ml.) or heat-inactivated mitochondria (5mg. of protein/ml.) were added as indicated. The heat-inactivated mitochondria had been pretreated for 5min. at 60°. In control systems mitochondria were incubated under similar conditions but without the addition of bacterial cells. Incubation was for 3hr. at 30° with vigorous shaking in a final volume of 2ml. The number of bacterial cells present in the assay systems was determined before and after incubation. Before acid precipitation the bacterial systems were supplemented with mitochondria (5mg. of protein/ml.). In Expt. 1 protein was precipitated with $HClO_4$ and extracted, and the radioactivity was measured in a thin-window gas-flow counter. In Expt. 2, after incubation the mixture was transferred to filter-paper disks and assayed as described in the Experimental section; radioactivity was measured by liquid-scintillation counting.

	Expt. 1			Expt. 2		
	No. of bacterial cells/ml.		Incorporation (counts/min./mg. of protein)	No. of bacterial cells/ml.		Incorporation (counts/min./mg. of protein)
	Before incubation	After incubation		Before incubation	After incubation	
Bacteria	4×10^3	4×10^3	0	10^4	$1{\cdot}3\times10^4$	15
Bacteria + mitochondria	4×10^3	$7{\cdot}5\times10^4$	32	10^4	3×10^4	85
Bacteria + heat-inactivated mitochondria	4×10^3	10^5	60	10^4	3×10^4	115
Mitochondria	—	<10	2	—	<10	0

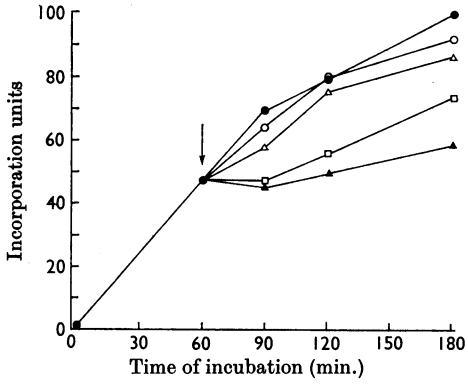

Fig. 3. Mitochondria were prepared in 0·25M-sucrose under non-sterile conditions. The suspension (2mg. of protein/ml.) was incubated in medium B with L-[^{14}C]leucine (0·25 μc/ml.) at 37° for the time indicated. At the arrow, 2,4-dinitrophenol was added to give the following concentrations: ▲, 0·2mM; □, 0·1mM; △, 0·02mM; ○, 0·01mM; ●, none (control). The results were obtained from three independent experiments, each experiment being run in duplicate. The results were calculated as percentages of the radioactivity obtained at 180min. of incubation. This incorporation unit was made equal to 100 in the Figure and corresponds to 450, 590, and 1080counts/min./mg. of protein respectively in the three experiments as measured by liquid-scintillation counting. The numbers of bacterial cells in the experiments were 2×10^4, $3{\cdot}5\times10^4$ and $6{\cdot}2\times10^4$ respectively.

dinitrophenol was studied with concentrations in the range 0·01–0·2mM. At concentrations as low as 0·01mM only a slight inhibition was observed. The rate of amino acid incorporation in the absence of 2,4-dinitrophenol was almost linear with time. The difference in the shape of the curve compared with the results shown in Fig. 2 was partially due to the uncontrolled phase of growth of the contaminating bacteria. A series of experiments was performed adding 2,4-dinitrophenol at the beginning of the incubation. The results are summarized in Table 8. The inhibition showed some variation, as indicated by the standard deviations from the mean values. The data obtained were compared with those found in the literature. Excluding the results of Kroon (1966), which show an exceptionally high inhibition at low concentrations of 2,4-dinitrophenol, there is a good correlation between the data presented here and those found earlier by others. These findings are further supported by the calculations of the statistical significance of our results.

The effect of *m*-chlorophenylhydrazone, another uncoupler of oxidative phosphorylation that acts at lower concentrations than 2,4-dinitrophenol, has been studied. At 0·1 μM an inhibition of amino acid incorporation of about 28% was observed. At this concentration mitochondrial functions are inhibited by about 40% (Heytler, 1963).

Table 8. *Effect of 2,4-dinitrophenol on the incorporation of amino acids into protein by mitochondrial preparations*

Mitochondria were prepared in 0·25 M-sucrose under non-sterile conditions. The suspension (2 mg. of protein/ml.) was incubated in medium B with L-[^{14}C]leucine (0·25 μc/ml.) for 2 hr. at 37° with shaking. 2,4-Dinitrophenol was added at the concentrations indicated. After incubation the proteins were precipitated on filter-paper disks and assayed as described in the Experimental section; radioactivity was measured by liquid-scintillation counting. In the first row results are given as means ± S.D., with in parentheses the numbers of independent experiments, each experiment being run in duplicate.

Reference	Source of mitochondrial preparation	Concn. of dinitrophenol (mM) ...	Inhibition of incorporation (%) 0·5	0·3	0·2	0·1	0·05	0·02	0·01
This paper	Rat liver		69·2 ± 16·1 (8)		48·3 ± 13·7 (12)	28·1 ± 8·4 (15)	16·8 ± 2·4 (5)	14·3 ± 3·0 (11)	12·3* (7)
Roodyn *et al.* (1961, 1965)	Rat liver		74 85		89				
Bronk (1963)	Rat liver				95 92				
Kalf (1963)	Rat heart			77					
Truman & Löw (1963)	Ox heart		73	71		21			
Kroon (1964, 1966)	Rat liver		81 76				91	68	50
Kroon (1963, 1964)	Ox heart		91 64 73 68						
Kroon (1964, 1965)	Ox heart (submitochondrial particles)		84 86						
	Rat liver (submitochondrial particles)		88						

* Two of the experiments showed an inhibition of 24·5% and two experiments showed no inhibition at all.

DISCUSSION

Under the conditions described isolated mitochondria from rat livers were unable to incorporate significant amounts of labelled amino acids into protein. An important condition was the virtual absence of bacteria from our preparations. Several procedures may be used for preparing mitochondria under sterile conditions. In previous experiments the whole liver was rinsed in 70% (v/v) ethanol before washings in sterile sucrose (Decken *et al.* 1966). It was found that this and other precautions previously employed were not needed to obtain mitochondrial preparations containing less than 10 bacteria/5 mg. of mitochondrial protein.

Microsomal contamination was kept at a minimum. Activity measurements of the microsomal enzyme glucose 6-phosphatase showed that the contamination was about 5%, calculated per unit of protein. This value is rather low (de Duve *et al.* 1955); yet, in several cases, it was sufficient to support a limited but ribonuclease-sensitive incorporation (Table 4).

The lack of correlation between the number of contaminating bacteria and the incorporating activity obtained has often been used as an argument against the significant contribution by bacteria to the incorporation of amino acid into protein by mitochondrial preparations. In accordance with such an argument the results presented in Table 6 and Fig. 2 strongly suggest that there is not necessarily a correlation between the rate of bacterial growth and the amino acid-incorporating activity.

It has been suggested by several authors (Roodyn *et al.* 1961, 1965; Truman & Korner, 1962; Bronk, 1963; Kroon, 1966) that a high phosphorylating capacity is needed to obtain an incorporation of amino acids into protein. It has been shown by the same authors that uncoupling agents inhibit the labelling of proteins. Since an insufficient supply of oxygen is rate-limiting for the incorporation (Roodyn, 1965), and 2,4-dinitrophenol abolishes respiratory control and increases the consumption of oxygen, the conditions for protein synthesis in the presence of this agent may no longer be optimum. In other words, the observed effect of 2,4-dinitro-

Table 9. *Comparison of data reported by other workers and in the present paper on conditions for amino acid incorporation by rat-liver mitochondrial preparations*

References given in the Table are as follows: (1) McLean *et al.* (1958), Table 5; (2) Roodyn (1962), Table 5; Roodyn *et al.* (1961), Fig. 8; Roodyn *et al.* (1962), Table 2; (3) Truman & Korner (1962), Fig. 5; (4) Bronk (1963), Table 1; (5) Kroon (1963), Table 1; Kroon (1964), Table 2; Kroon (1965), Fig. 1; (6) present paper. Values for μmoles incorporated/mg. of protein were recalculated from the data available. Radioactive amino acids used were leucine (ref. 1, 3–6) or valine (ref. 2). The pH of the incubation medium was 7·4 (ref. 1, 3–5), 7·0–7·2 (ref. 2) or 7·5 (ref. 6). Incubation was in air (ref. 1, 3–6) or in O_2 (ref. 2).

Conditions	References ... (1)	(2)	(3)	(4)	(5)	(6)
Rats	200g.	100–120g. (starved)	200g.		120–125g. (starved)	120–150g. (non-starved)
Mitochondria isolated in	0·25M-Sucrose	0·3M-Sucrose–2mM-EDTA–0·03M-nicotinamide, pH 7·1.	0·25M-Sucrose	0·25M-Sucrose	0·25M-Sucrose	0·25M-Sucrose ± 2mM-EDTA
First centrifugation of liver homogenate	10min., 700*g*	15min., 700*g*	10min., 600*g*	10min., 700*g*	10min., 900*g*	10min., 500*g*
Second centrifugation of liver homogenate	10min., 5000*g*	10min., 8000*g*	10min., 5000*g*	10min., 5000*g*	10min., 4500*g*	20min., 5000*g*
Substrate and cofactors added to incubation mixture	Succinate–AMP–cytochrome *c*	Succinate–AMP–nicotinamide–NAD–EDTA	Succinate–AMP	Succinate–nicotinamide–NAD	ADP–EDTA	None or succinate–ADP
Activity of ^{14}C-labelled amino acid	5×10^5 counts/min./0·31 μmole	1mc/17·9mg.		4·03mc/m-mole	10mc/m-mole	20mc/m-mole
Mitochondrial protein added	2–3mg./ml.	3–6mg./ml.	6mg./ml.	3·5mg./ml.	2–3mg./ml.	5mg./ml.
Time and temp.	20min., 37°	60–90min., 30°	60min., 37°	60min., 37°	120min., 30° or 37°	120min., 37°
Incorporated radioactivity/mg. of protein	19·1 counts/min.	48 $\mu\mu$c 66·0 $\mu\mu$c 23·5 $\mu\mu$c	6 counts/min.	49 counts/min.	353 counts/min. 316 counts/min. 280 counts/min.	0–5 counts/min.
Recalculation to $\mu\mu$moles incorporated/mg. of protein	11·9	3·6–10·4				0–0·64

phenol in our systems containing mitochondria and bacteria might be due to an insufficient supply of oxygen to the bacteria inhibiting their synthetic activity. On the other hand, Simon *et al.* (1966) have reported a direct effect of 2,4-dinitrophenol on protein synthesis in *E. coli*.

A wide range of rates of incorporation of amino acids into protein by mitochondrial preparations has been reported. In an attempt to elucidate the reason for the variability a comparison was made between the conditions used and results obtained in several Laboratories. Within these data (summarized in Table 9) small variations in experimental conditions are apparent. The weight of the animals varied between 100 and 200g.; both starved and non-starved rats were used; media and centrifugation forces were in the range normally employed to isolate mitochondria; the amount of mitochondria added, the final pH and the gas phase during incubation were dissimilar; and substrates and cofactors added to the incubation medium were not the same in all cases. The highest incorporation was obtained in the absence of oxidizable substrate (Kroon, 1963; 1964, 1965), although Truman & Korner (1962) found a slight stimulation (not exceeding 6counts/min.) by succinate in their system. Under our conditions, however, substrate seemed to have but little effect on the incorporating activity.

The findings on the effect of oxidizable substrate, although apparently inconsistent, together with the other variations in technique mentioned, may account for the differences in results reported by the various authors. On the other hand, the fact that the absence of bacteria from our systems decreased the incorporating activity to an insignificant rate makes it likely that the discrepancies in the literature could well be explained in terms of differences in bacterial number and species.

In summary, our results suggest that isolated liver mitochondria obtained from adult rats and free from bacteria do not show significant amino acid incorporation. As outlined above, this is not in agreement with what has been reported by other workers. Although our preparations were fully active in oxidative phosphorylation the techniques employed may be unfavourable to the conditions of protein synthesis.

We thank Professor O. Lindberg for his interest and advice. We are greatly indebted to Dr G. Lundblad for identifying bacterial strains. This work was supported by joint grants from the Swedish Medical Research Council and the Swedish Cancer Society.

REFERENCES

Bronk, J. R. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 524.
Chance, B. & Williams, G. R. (1955). *J. biol. Chem.* **217**, 383.
Decken, A. von der & Campbell, P. N. (1964). *Biochem. J.* **91**, 195.
Decken, A. von der, Löw, H. & Sandell, S. (1966). In *Biochim. biophys. Acta Library vol. 7: Regulation of Metabolic Processes in Mitochondria*, p. 415. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Ernster, L. & Löw, H. (1955). *Exp. Cell Res.* Suppl. 3, 133.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Heytler, P. G. (1963). *Biochemistry*, **2**, 357.
Kalf, G. F. (1963). *Arch. Biochem. Biophys.* **101**, 350.
King, E. J., Abul-Fadl, M. A. N. & Walker, P. G. (1951). *J. clin. Path.* **4**, 85.
Kroon, A. M. (1963). *Biochim. biophys. Acta*, **72**, 391.
Kroon, A. M. (1964). *Biochim. biophys. Acta*, **91**, 145.
Kroon, A. M. (1965). *Biochim. biophys. Acta*, **108**, 275.
Kroon, A. M. (1966). *Protein Synthesis in Mitochondria*, pp. 1–48. Hoorn: Drukkerij Noordholland N.V.
Lamborg, M. R. & Zamecnik, P. C. (1960). *Biochim. biophys. Acta*, **42**, 206.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McLean, J. R., Cohn, G. L., Brandt, I. K. & Simpson, M. V. (1958). *J. biol. Chem.* **233**, 657.
Mans, R. J. & Novelli, G. D. (1961). *Arch. Biochem. Biophys.* **94**, 48.
Roodyn, D. B. (1962). *Biochem. J.* **85**, 177.
Roodyn, D. B. (1965). *Biochem. J.* **97**, 782.
Roodyn, D. B., Freeman, K. B. & Tata, J. R. (1965). *Biochem. J.* **94**, 628.
Roodyn, D. B., Reis, P. J. & Work, T. S. (1961). *Biochem. J.* **80**, 9.
Roodyn, D. B., Suttie, J. W. & Work, T. S. (1962). *Biochem. J.* **83**, 29.
Sandell, S., Löw, H. & Decken, A. von der (1966). *Abstr. 3rd Meet. Fed. Europ. biochem. Soc., Warsaw*, p. 167. London: Academic Press (Inc.) Ltd.
Simon, E. J., Van Praag, D. & Aronson, F. L. (1966). *Molec. Pharmacol.* **2**, 43.
Suttie, J. W. (1962). *Biochem. J.* **84**, 382.
Truman, D. E. S. (1964). *Biochem. J.* **91**, 59.
Truman, D. E. S. & Korner, A. (1962). *Biochem. J.* **83**, 588.
Truman, D. E. S. & Löw, H. (1963). *Exp. Cell Res.* **31**, 230.
Yarmolinsky, M. B. & De La Haba, G. L. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1721.