Lightbown, J. W. & Kogut, M. (1959). *Biochem. J.* **73**, 14P.
Mackie, T. J. & McCartney, J. E. (1949). *Handbook of Practical Bacteriology*, p. 145. Edinburgh: E. and S. Livingstone Ltd.
Margoliash, E. (1954*a*). *Biochem. J.* **56**, 529.
Margoliash, E. (1954*b*). *Biochem. J.* **56**, 535.
Pullman, M. E., Colowick, S. P. & Kaplan, N. O. (1952). *J. biol. Chem.* **194**, 593.
Rieche, A. & Hitz, F. (1929). *Ber. dtsch. chem. Ges.* **62**, 2458.
Sadana, J. C. & McElroy, W. D. (1957). *Arch. Biochem. Biophys.* **67**, 16.
Sands, R. H. & Beinert, H. (1960). *Biochem. biophys. Res. Commun.* **3**, 47.
Smith, F. G. & Stotz, E. (1949). *J. biol. Chem.* **179**, 865.
Smith, L. & Baltscheffsky, M. (1959). *J. biol. Chem.* **234**, 1575.
Tanigushi, S. & Itagaki, E. (1960). *Biochim. biophys. Acta*, **44**, 263.
Venkataraman, B. & Fraenkel, G. K. (1955). *J. Amer. chem. Soc.* **77**, 2707.
Vernon, L. P. (1959). *J. biol. Chem.* **234**, 1883.
Vernon, L. P. & Ash, O. K. (1959). *J. biol. Chem.* **234**, 1878.
Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.
Whitby, L. G. (1953). *Biochem. J.* **54**, 437.
Yamazaki, I., Mason, H. S. & Piette, L. (1959). *Biochem. biophys. Res. Commun.* **1**, 336.

Biochem. J. (1962) **84**, 382

The Existence of Two Routes for Incorporation of Amino Acids into Protein of Isolated Rat-Liver Mitochondria

BY J. W. SUTTIE*
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 15 *January* 1962)

It has been demonstrated (Roodyn, Reis & Work, 1961; Roodyn, Suttie & Work, 1962) that isolated, washed, rat-liver mitochondria can incorporate amino acids into protein. Amino acid incorporation into mitochondrial protein is dependent on the maintenance of oxidative phosphorylation and, under optimum conditions (Roodyn *et al.* 1961), the rate of incorporation was linear for about 1 hr. Incorporation could, however, be maintained for longer periods if the medium were changed at regular intervals.

None of the many changes of incubation conditions which were tried led to any increase in the initial rate of synthesis; it seemed worth while, therefore, to try to extend the period of synthesis beyond the 3 hr. already achieved, in the hope of demonstrating net synthesis of protein. It was possible to extend the period of incorporation by frequent changes of medium; unfortunately, there was a progressive change in the characteristics of the incorporation system, so that it was no longer dependent on oxidative phosphorylation. This secondary process of amino acid incorporation was completely resistant to various inhibitors that had prevented incorporation in the primary, phosphorylation-dependent, system. Evidence is now presented which strongly suggests that the secondary, non-phosphorylative, incorporation is due to transpeptidation.

* Present address: Department of Biochemistry, University of Wisconsin, Madison 6, Wis., U.S.A.

METHODS

Isolation of mitochondria. Mitochondria were isolated from rat liver as described by Roodyn *et al.* (1961). They were washed with four changes of sucrose–EDTA–nicotinamide as described previously, and the pellet from the fourth wash was suspended either in 0·25 M-sucrose or in the sucrose–EDTA–nicotinamide mixture of Roodyn *et al.* (1961).

Incubation conditions. For experiments on the phosphorylation-dependent incorporation, the incubation conditions were those given by Roodyn *et al.* (1961) with 'medium B' [0·1 M-sucrose, 0·04 M-KCl, 8 mM-$MgSO_4$, 1·3 mM-EDTA (disodium salt), 0·02 M-nicotinamide, 0·01 M-potassium succinate, 0·016 M-potassium phosphate, 4 mM-AMP, 0·5 mM-NAD, 50 μg. of synthetic amino acid mixture/ml.; pH adjusted to 7–7·2 with KOH]. In the experiments where non-phosphorylative incorporation was examined, the mitochondria were incubated in a medium containing only sucrose (0·12 M) and tris–HCl buffer, pH 7·5 (0·05 M with respect to tris). In all experiments penicillin G (100–150 units/ml.) was present.

Bacterial contamination. After incubation a sample of the mitochondrial suspension was centrifuged at 8000*g* for 10 min. to sediment both bacteria and mitochondria. The pellet was resuspended in fresh 0·25 M-sucrose and samples were plated on blood–agar. Colony counts were made after 24 hr. at 37°.

Treatment of radioactive proteins

All proteins were washed and extracted by the standard method of Simkin & Work (1957) before radioactivities were determined.

Removal of non-peptide bound amino acid. As a check

that all the radioactivity was held in peptide linkage, washed proteins were treated further with performic acid, ninhydrin or thioglycollic acid. For the performic acid treatment, 10 mg. of protein was dissolved in 1 ml. of formic acid, and 0·2 ml. of hydrogen peroxide (30%, w/v) was added. After 30 min. at room temperature the solution was diluted with water and protein was precipitated with 5% (w/v) trichloroacetic acid. For ninhydrin treatment, 10 mg. of protein was suspended in 2 ml. of 0·2M-sodium citrate buffer, pH 5, and heated at 100° with a mixture of 0·4 ml. of ninhydrin–methylCellosolve and 1 ml. of KCN–methylCellosolve (Yemm & Cocking, 1955). Protein was recovered by precipitation with 5% (w/v) trichloroacetic acid. For thioglycollic acid treatment, 10 mg. of protein was mixed with 0·2 ml. of thioglycollic acid and 0·8 ml. of water. After 1 hr. at room temperature, 0·7 ml. of 5N-NaOH was added and the protein was precipitated with excess of 5% (w/v) trichloroacetic acid.

Distribution of radioactivity between N-terminal amino acids, basic amino acids and non-basic amino acids. To assess whether incorporated radioactive amino acids were uniformly distributed throughout radioactive protein, the following technique was used. A sample (5 mg.) of the fully washed radioactive protein was allowed to react with 0·1 ml. of fluorodinitrobenzene at room temperature by shaking with 2 ml. of ethanol, 1 ml. of water and 0·1 g. of $NaHCO_3$. After 3 hr. the mixture was diluted with 5% (w/v) trichloroacetic acid, and the precipitate was washed with acetone and ether and dried. The dried protein was hydrolysed at 105° with 6N-HCl (16 ml.) and the *N*-terminal dinitrophenyl-amino acids were extracted into ether. Basic dinitrophenyl-amino acids were then extracted into butan-1-ol and amino acids left in the aqueous phase were adsorbed on Zeo-Karb 225 (H^+ form) and eluted with ammonia. The various fractions collected were plated on aluminium planchets and radioactivity was determined at infinite thinness.

Removal of C-terminal amino acids. A method based on the procedure of Waley & Watson (1951) was used to remove *C*-terminal residues selectively. A sample (4 mg.) of washed radioactive protein was mixed with 1·5 ml. of acetic anhydride and 0·2 ml. of formic acid. Ammonium thiocyanate (4 mg.) was added and the solution heated at 50° for 5 hr. Solvent was removed *in vacuo* over KOH and the residue suspended in 5% (w/v) trichloroacetic acid. The precipitated protein was dissolved in 1 ml. of N-NaOH and heated at 50° for 5 min. Excess of 5% (w/v) trichloroacetic acid was added and the precipitated protein washed once with 5% (w/v) trichloroacetic acid, twice with acetone and once with ether. The dried powder was dissolved in formic acid and plated on aluminium planchets for assay of radioactivity.

Radioactive chemicals and counting methods. All experiments were carried out either with L-[^{14}C]valine (1 mc/17·9 mg.) or with a hydrolysate of *Chlorella* protein (1 mc/5·6 mg.), both uniformly labelled with ^{14}C. The protein hydrolysate was purified by the method of Simkin (1958). Radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks.

All assays of radioactivity were performed with a thin-window Geiger tube which was standardized with a poly-(methyl methacrylate) disk of specific activity 1 μc/g.

Table 1. *Effect of preincubation on the energy dependence of amino acid incorporation into protein of isolated rat-liver mitochondria*

In Expt. 1, mitochondria were preincubated in medium B (see text) for periods of 45 min. The incorporation period was 60 min. with 0·33 μc of ^{14}C-labelled *Chlorella*-protein hydrolysate added per ml. In Expt. 2, the same medium was used, but the preincubations were for 60 min. and 0·2 μc of [^{14}C]valine was added per ml. for the 60 min. incorporation periods.

	Radioactivity (μmc/g.)			
	Expt. 1		Expt. 2	
No. of pre-incubations	Succinate	No succinate	Succinate	No succinate
Fresh	134	3	38	3
1	—	—	25	7·5
2	105	36	20	15
3	—	—	31	25
4	—	—	44	37
5	190	125	—	—

RESULTS

When washed rat-liver mitochondria were incubated with either [^{14}C]valine or ^{14}C-labelled *Chlorella*-protein hydrolysate in medium B of Roodyn *et al.* (1961), incorporation was almost complete in 90 min. But when a similar mitochondrial preparation was incubated in this medium for 1 hr. and the mitochondria were then sedimented at 8000*g*, resuspended in fresh medium and reincubated, the process could be repeated for up to 5 hr. without any significant diminution in the capacity to incorporate radioactive amino acid into protein. However, the incorporation of amino acids into preincubated mitochondria was no longer entirely dependent on oxidative phosphorylation. The progressive change in the character of the incorporation system is clearly shown in Table 1 and applies equally with either a single amino acid or a complete *Chlorella*-protein hydrolysate as the radioactive tracer.

Requirements for incorporation of amino acids into preincubated mitochondria. None of the cofactors present in the normal incubation medium was essential for the non-phosphorylative incorporation. Thus, when mitochondria were preincubated in several changes of buffered sucrose (Table 2), there was a development of the capacity for non-phosphorylative incorporation which in some experiments increased 6–7-fold during the course of four preincubations. However, when the medium was not changed at regular intervals there was no significant development of non-phosphorylative incorporation even after 5 hr. at 30°.

Possible effects of bacterial contamination. In any system where the incubation time is several hours the possibility of protein synthesis by contaminating bacteria must be considered. However, the

non-phosphorylative incorporation was unaffected by penicillin, chloramphenicol, vancomycin, ristomycin or puromycin (Table 2): this resistance to antibiotics strongly indicated that bacteria were not involved. As an additional check, samples were taken from the mitochondrial preparation after the first cycle of incubation and sedimentation and also after four to six periods of incubation. Each sample was assayed for viable bacteria as described in the Methods section. Table 3 shows that there is no relationship between the number of bacteria and the final radioactivity of the mitochondrial protein. These results also indicate the extreme variation in radioactivity observed in different experiments with the preincubated system. It was not possible to correlate the variation in activity with any of the changes made in the medium. The choice of medium B or buffered sucrose as a preincubation medium did not consistently affect the results.

Mechanism of non-phosphorylative incorporation. In testing the effect of various inhibitors, it was noticed that hydroxylamine in low concentrations (10 mM) almost completely inhibited non-phosphorylative incorporation. This observation was consistent with the idea that the process might be due to transpeptidation, in which hydroxylamine could compete effectively with amino acids. To confirm this idea it was necessary to show, first, that the amino acids were in true peptide linkage, and, secondly, that labelled peptide chains were more heavily labelled at or near the carboxyl end than elsewhere. Performic acid, thioglycollic acid and ninhydrin, used as described (see Methods section), had negligible effects in releasing radioactivity. In this respect the labelling has the character of true peptide linkage. Protein labelled with ^{14}C-labelled *Chlorella*-protein hydrolysate by the non-phosphorylative process was allowed to react with fluorodinitrobenzene and then hydrolysed (see Methods section). The distribution of radioactivity in the hydrolysate (Table 4) showed that the radioactive amino acids were not *N*-terminal, and that after hydrolysis much of the radioactivity could be recovered as compounds behaving as amino acids on Zeo-Karb 225.

The effectiveness of hydroxylamine as an inhibitor and the absence of *N*-terminal labelling suggested that the process under investigation might be one that would lead to preferential incorporation of the radioactive tracer into *C*-terminal positions of the mitochondrial protein. As a check on this possibility, radioactive mitochondrial protein prepared by the non-phosphorylative reaction was compared with mitochondrial protein labelled either in the intact animal or *in vitro* by the succinate-dependent phosphorylative process described by Roodyn *et al.* (1961). All

Table 2. *Effect of preincubation on characteristics of amino acid incorporation into isolated rat-liver mitochondria*

Mitochondria were preincubated for five periods of 45 min. in medium B (see text). The incorporation period was 60 min. with 0·33 μc of ^{14}C-labelled *Chlorella*-protein hydrolysate added per ml.

System	Radioactivity (μmc/g.) Fresh mitochondria	Preincubated 5 times
Control	248	965
Succinate omitted	41	815
Chloramphenicol (10 μg./ml.) added	115	782
Succinate omitted; chloramphenicol (10 μg./ml.) added	40	812
Vancomycin + ristomycin (10 μg./ml. each) added	—	995
Succinate omitted; vancomycin + ristomycin (10 μg./ml. each) added	—	932

Table 3. *Relationship between bacterial contamination and amino acid incorporation*

Mitochondria were preincubated in medium B (see text) or buffered sucrose (see text) at 30° for periods of 45 or 60 min. The incorporation period was 60 min. in all cases. [^{14}C]Valine was added at a concentration of 0·2 μc/ml., and the ^{14}C-labelled *Chlorella*-protein-hydrolysate concentration was 0·33 μc/ml. in medium B and 0·5 μc/ml. in buffered sucrose. —, No bacterial counts made.

Expt.	Medium	Label used	No. of preincubations	Colonies/ml. First preincubation	Colonies/ml. Final incubation	Radioactivity (μmc/g.)
1	B	*Chlorella*	5	1×10^4	5×10^4	190
2	B	*Chlorella*	5	5×10^4	1×10^4	965
3	B	Valine	4	—	7×10^4	9
4	B	Valine	6	1×10^4	2×10^4	83
5	B	Valine	5	4×10^4	2×10^4	14
6	Buffered sucrose	*Chlorella*	4	3×10^4	4×10^4	134
7	Buffered sucrose	*Chlorella*	4	—	5×10^4	25
8	Buffered sucrose	*Chlorella*	4	—	1×10^4	58
9	Buffered sucrose	*Chlorella*	4	—	3×10^4	35

Table 4. *Distribution of radioactivity in hydrolysed protein*

Mitochondrial protein (5 mg.), isolated from a buffered-sucrose incubation with ^{14}C-labelled *Chlorella*-protein hydrolysate, was hydrolysed and treated as described in the text.

Fraction	Radioactivity (total counts/min.)	
N-Terminal amino acids	2	
Non-*N*-terminal amino acids	150	
Basic amino acid derivatives (mono DNP-)	25	145
Water wash of resin	35	
Ammonia eluate from resin	85	
Humin	<5	

Table 5. *Effect of chemical removal of the* C*-terminal amino acid*

The three types of mitochondrial proteins were treated under identical conditions as described in the text, and the results expressed as the percentage of the untreated specific radioactivity; the values given are means ± S.E.M., with the numbers of determinations in parentheses. The energy-dependent system *in vitro* was that described by Roodyn *et al.* (1961).

System	Percentage of untreated specific activity
Mitochondrial protein labelled *in vivo*	108 ± 2 (5)
Energy-dependent system labelled *in vitro*	95 ± 2 (3)
Non-energy-dependent system labelled *in vitro*	72 ± 4 (6)*

* Mitochondria preincubated with four changes of buffered sucrose and labelled with ^{14}C-labelled *Chlorella*-protein hydrolysate as in Table 3.

three types of protein were degraded by the isothiocyanate method (cf. Greenstein & Winitz, 1961) which selectively removes at least some of the *C*-terminal amino acids from peptide chains. The results of a series of such experiments (Table 5) show that mitochondria labelled *in vivo*, or *in vitro* by the succinate-dependent reaction of Roodyn *et al.* (1961), contained negligible quantities of the added tracer (^{14}C-labelled *Chlorella*-protein hydrolysate) in the *C*-terminal position, whereas nearly 30 % of the radioactivity of material formed during the non-phosphorylative reaction was lost in this partial removal of the *C*-terminal amino acids.

DISCUSSION

The results of the present investigation indicate that in mitochondria two distinct types of amino acid incorporation can take place *in vitro*. The succinate-dependent amino acid incorporation described by Roodyn *et al.* (1961) has all the characteristics of true protein synthesis and leads to the formation of a radioactive insoluble 'structural' lipoprotein (D. B. Roodyn, personal communication). Preparations of liver mitochondria do, however, contain catheptic enzymes. In a fresh preparation these enzymes are in a latent form and are probably contained in particles other than mitochondria, the so-called lysosomes (de Duve & Berthet, 1954; de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). The process of periodic transfer of mitochondria to fresh medium apparently tends to preserve their capacity for oxidative phosphorylation (Roodyn & Suttie, 1961), but at the same time it also leads to a gradual release of enzymes capable of catalysing exchange between preformed protein and amino acids present in the medium.

There is much evidence in the literature for the formation of protein-like substances ('plastein') on incubation of amino acids with proteolytic enzymes (Haurowitz, 1950). It is also well established (Zioudrou, Fujii & Fruton, 1958) that transpeptidation can occur between glycyltyrosine amide and insulin or between tyrosine amide and mitochondrial protein, but it has not hitherto been realized that similar transpeptidations may take place between protein and free amino acids. The reaction could account for the labelling observed, and presumably takes the form:

$$\text{Protein}\cdot\text{CO}\cdot\text{NH}\cdot\text{CHR}\cdot\text{CO}\cdot\text{R}' + \text{HN}_2\cdot\text{X} \rightleftharpoons \text{Protein}\cdot\text{CO}\cdot\text{NH}\cdot\text{X} + \text{NH}_2\cdot\text{CHR}\cdot\text{CO}\cdot\text{R}'$$

If this is the process occurring in the mitochondrial system, it is understandable that hydroxylamine should be an extremely effective inhibitor since the effective concentration of NH_2X provided by hydroxylamine would be very much higher than could be provided by amino acids with their predominantly zwitterion structure.

The present investigations must be taken as a warning that amino acid incorporation by itself is not an adequate criterion of protein synthesis. However, only a negligible proportion of the incorporation observed *in vitro* in fresh mitochondria (Roodyn *et al.* 1961) is due to this type of reaction.

SUMMARY

1. When isolated, washed, rat-liver mitochondria are preincubated at 30° with successive changes of medium, labelled amino acids are incorporated into protein in a manner which is not dependent on oxidative phosphorylation.

2. This system is not affected by puromycin or chloramphenicol, but is strongly inhibited by hydroxylamine.

3. Evidence is presented to indicate that the labelling is concentrated at the *C*-terminal groups of the mitochondrial proteins, and it is suggested that a transpeptidation catalysed by slowly-activated catheptic enzymes is responsible for the labelling.

The author is indebted to Dr T. S. Work for valuable discussions during the course of the work, and to Miss A. Pearson for technical assistance. The author held a Post-Doctoral Fellowship from the National Science Foundation during the course of this work.

REFERENCES

de Duve, C. & Berthet, J. (1954). *Int. Rev. Cytol.* **3**, 225.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Greenstein, J. P. & Winitz, M. (1961). *Chemistry of the Amino Acids*, vol. 2, p. 1576. New York: J. Wiley and Sons Inc.
Haurowitz, F. (1950). *Chemistry and Biology of Proteins*, pp. 326, 327. New York: Academic Press Inc.
Roodyn, D. B., Reis, P. J. & Work, T. S. (1961). *Biochem. J.* **80**, 9.
Roodyn, D. B. & Suttie, J. W. (1961). *Biochim. biophys. Acta*, **52**, 376.
Roodyn, D. B., Suttie, J. W. & Work, T. S. (1962). *Biochem. J.* **83**, 29.
Simkin, J. L. (1958). *Biochem. J.* **70**, 305.
Simkin, J. L. & Work, T. S. (1957). *Biochem. J.* **67**, 617.
Waley, S. G. & Watson, J. (1951). *J. chem. Soc.* p. 2394.
Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.
Zioudrou, C., Fujii, S. & Fruton, J. S. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 439.

Biochem. J. (1962) **84**, 386

Unsaponifiable Constituents of Blowfly Larvae (*Calliphora erythrocephala*)

BY D. L. LAIDMAN* AND R. A. MORTON
Department of Biochemistry, University of Liverpool

(*Received* 24 *January* 1962)

The initial object of the present work was to find out whether ubiquinone (coenzyme Q) occurred in the insect world, and, if it did, to identify the particular isoprenologue. The larval form of the blowfly was chosen as an easily-accessible raw material obtainable from firms who sell the 'gentles' as bait to anglers. To attain the initial goal it was necessary to prepare the total unsaponifiable fraction and to carry out a separation of the various constituents. In so doing the scope of the investigation was widened.

MATERIALS AND METHODS

Blowfly larvae. These were bought from the 'gentles' farm of Ellis Ashurst Ltd., Conisburgh, Yorks. They arrived packed in sawdust and shavings and were too active to be easily picked out. The container and its contents were therefore cooled to $-30°$ for about 1 hr. so that the dead larvae and sawdust could be more readily separated.

Saponification. The larvae (free from sawdust) were homogenized in a mechanical blender. Aq. KOH [60% (w/v); 0·5 ml./g. of tissue] and methanolic pyrogallol (0·3%; 1 ml./g. of tissue) were mixed and then added to the homogenate. The mixture was heated under reflux for 1 hr. on a water bath (Mervyn & Morton, 1959; Mervyn, 1957).

After being cooled, the solution was diluted with water (2 vol.) and extracted five times with diethyl ether (freshly redistilled from reduced iron). The combined extracts were washed until free from alkali and dried (over Na_2SO_4), and the ether was removed under nitrogen. The unsaponifiable matter, and fractions derived from it, were stored in the dark at $-20°$ as far as possible.

Alumina. Grade O alumina (P. Spence and Co., Widnes, Lancs.) was acid-washed, heated to 400° and partially deactivated to Brockmann Grade 3 by addition of water to a slurry made with light petroleum (b.p. 40–60°).

Adsorption chromatography. The unsaponifiable material was chromatographed first on alumina and some fractions were re-chromatographed on magnesia (Laidman, Morton, Paterson & Pennock, 1960). Details are shown in Table 1.

Spectroscopy. All the fractions were examined in cyclohexane in the visible and ultraviolet regions (in a Unicam SP. 500 instrument), and infrared spectra of some fractions were measured (in the Infracord spectrophotometer of Perkin–Elmer) either as films or in potassium bromide disks.

Reversed-phase chromatography on paper. The system petroleum jelly-treated paper (stationary phase)–2·5% (v/v) water in dimethylformamide (mobile phase) (Linn *et al.* 1959) was used, and pure specimens of ubiquinones were used for calibration purposes. The method was made semiquantitative by extracting separated isoprenologues from sections of paper after development, followed by ultraviolet spectrophotometry on the extracts.

Determination of ubiquinone. Provided that the absorption curve showed the absence of significant irrelevant absorption the molar concentration could be determined directly by measuring the intensity of absorption at 272 mμ in cyclohexane. When there was too much irrelevant absorption for this procedure to be valid, the quinone was reduced by KBH_4, and the change in E at 272 mμ was measured (Crane, Lester, Widmer & Hatefi, 1959).

* Holder of an Agricultural Research Council Studentship.