Further Observations on the Production of Amino Acids by Rat-Liver Mitochondria and other Subcellular Fractions

By K. G. M. M. ALBERTI AND W. BARTLEY*

Department of Biochemistry, University of Oxford

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1. Rat-liver mitochondria showed a decrease in amino acid production after preparation in 0.25M-sucrose containing EDTA (1mM), but an increase in water content. When EDTA was replaced by Mn^{2+} (1mM) or succinate (1mM), both amino acid production and water content were lowered, whereas preparation in 0.9% potassium chloride caused an increase in both. 2. Amino acid production by rat-liver homogenates prepared in 0.9% potassium chloride or 0.25M-sucrose was similar ($q_{\text{amino acid}}$ 0.047 and 0.042 respectively aerobically). After freezing-and-thawing $q_{\text{amino acid}}$ values were approximately doubled, and approached that of a homogenate prepared in water. 3. All cations tested inhibited amino acid production by mitochondria, Hg^{2+} and Zn^{2+} being the most effective in tris-hydrochloric acid buffer. In phosphate buffer Mg^{2+} and Mn^{2+} had no effect. Of the anions tested only pyrophosphate and arsenate had any inhibitory effect at final concn. 1mM. 4. Iodobenzoate (1mM) and *p*-chloromercuribenzenesulphonate (1mM) inhibited mitochondrial amino acid production by 70–80%, whereas soya-bean trypsin inhibitor, EDTA and di-isopropyl phosphorofluoridate inhibited by a maximum of 30%. Respiratory inhibitors had no effect. 5. Rat-liver homogenate and subcellular fractions each showed an individual pattern of inhibition when a series of inhibitors was tested. 6. Amino acid production by mitochondria was decreased by up to 50% in the presence of oxidizable substrate, apart from α -glycerophosphate and palmitate, which had no effect. CoA stimulated amino acid production in tris-hydrochloric acid but not in phosphate buffer, α -oxoglutarate abolishing the stimulation. 7. Cysteine and glutathione stimulated amino acid production by whole mitochondria by 30%, but only reduced glutathione stimulated production in broken mitochondria. 8. Adrenocorticotrophic hormone and growth hormone stimulated mitochondrial amino acid production by 21–24%, whereas insulin inhibited production by 25%. 9. Coupled oxidative phosphorylation increased amino acid production by up to 154% at 25° and 40°. The increase was abolished by 2,4-dinitrophenol. 10. Amino acid incorporation in mitochondria was accompanied by an increase in amino acid production, both being decreased by chloramphenicol. 11. Mitochondrial production of ninhydrin-positive material was increased in the presence of albumin. The biggest increase was noted for the soluble fraction of broken mitochondria. No increase was found in the presence of ^{14}C -labelled algal protein or denatured mitochondrial protein.

A previous paper (Alberti & Bartley, 1963) showed that cell organelles from a variety of tissues and species liberated amino acids in substantial amounts (0.1–0.4 μ mole of amino acid/mg. dry wt./hr.) when incubated at 40° and pH 7.4. The presence of acid cathepsins has been known for some time in kidney, spleen and liver, but little is known of the neutral proteinases.

Penn (1961) described a CoA-dependent neutral

proteinase in rat-liver mitochondria breaking down endogenous protein, and Waley & van Heyningen (1962) showed the presence of a neutral proteinase in rat-liver homogenates. Albumin hydrolysis by rat-liver mitochondria at neutral pH has also been shown by Penn (1960).

The present paper describes the effect of different conditions on the neutral proteinases of the liver, with particular reference to the liberation of amino acids from mitochondria that are simultaneously incorporating labelled amino acids into their protein.

* Present address: Department of Biochemistry, University of Sheffield.

MATERIALS AND METHODS

Special chemicals. The ^{14}C -labelled algal-protein hydrolysate ($30\mu\text{C}/\text{mg.}$) and ^{14}C -labelled algal protein ($30\mu\text{C}/\text{mg.}$) were obtained from The Radiochemical Centre, Amersham, Bucks. Ox growth hormone, prepared by the method of Wilhelmi, Fishman & Russell (1948), and ox adrenocorticotrophic hormone were gifts to Professor H. A. Krebs from Professor F. G. Young. Insulin was obtained from Boots Pure Drug Co., Nottingham; thyroxine, GSH and EDTA were from British Drug Houses Ltd., Poole, Dorset; soya-bean trypsin inhibitor was from L. Light and Co. Ltd., Colnbrook, Bucks.; hexokinase (type III, practical grade) and *p*-chloromercuribenzenesulphonic acid were from Sigma Chemical Co., St Louis, Mo., U.S.A.; chloramphenicol (chloromycetin) was from Parke, Davis and Co., Hounslow, Middlesex; ADP and ATP were from Zellstoff-fabrik, Waldhof, Pharmazeutische Abteilung, Wiesbaden, Germany; CoA, AMP and NAD were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; iodosobenzoic acid and di-isopropyl phosphorofluoridate (DFP) were gifts from Dr S. G. Waley and Dr W. N. Aldridge respectively. Other chemicals were commercial products of A. R. standard where available.

Experimental animals. Male rats of the white Wistar strain, weighing 200–250 g., were used.

Preparation of rat-liver subcellular fractions. These were prepared as described by Alberti & Bartley (1963). Disrupted fractions were prepared by freezing-and-thawing seven times in liquid air.

Preparation media. Chilled 0.25 M-sucrose was used to prepare normal 'sucrose mitochondria'; 0.25 M-sucrose media containing MnCl_2 (1 mM), succinate (1 mM) or EDTA (1 mM) were also used where stated. Chilled 0.9% KCl was used for the preparation of 'potassium chloride mitochondria'.

Resuspension media. After the final mitochondrial pellet was obtained from the preparation, the mitochondria were either resuspended in the required volume of 0.25 M-sucrose or, where indicated, in one of the other 'preparation' media. Sometimes the pellet was washed several times in one of the resuspension media. The number of washings is indicated in the text.

Incubations. Unless otherwise stated, conditions were as described by Alberti & Bartley (1963). The total volume in the Warburg flasks was normally 4 ml., 1 ml. being either 0.1 M-potassium phosphate buffer, pH 7.4, or 0.1 M-tris-HCl buffer, pH 7.4.

In experiments to test the effect of oxidative phosphorylation on amino acid production, several different media were used. These are described in the legends to the appropriate Tables. Similarly, the medium used in experiments to show the uptake of ^{14}C -labelled amino acids is described in Table 11.

In all experiments where ninhydrin-positive substances were added, incubations were made with media containing all components except mitochondria, and the values of amino acids found in these tests were used as blank values for the incubations with mitochondria.

Amino acid extraction. The reactions were stopped and the amino acids extracted by the addition of 2 N-perchloric acid (final concn. 0.4–0.7 N). The precipitated protein was removed by centrifuging, and the perchloric acid removed from the supernatant by precipitation with 2 N-KOH at 0°.

Chemical estimations. Amino acids were estimated by the method of Rosen (1957), as adapted by Alberti & Bartley (1963). When metal ions were present in the samples to be analysed, 0.5 ml. of aq. 0.3% EDTA was added to the samples before making up the volume to 1 ml. with water, as otherwise colour yields were depressed. Even with EDTA present, Hg^{2+} still depressed the colour yield and therefore, in samples containing Hg^{2+} , H_2S was passed through the solution for 5 min., followed by N_2 to remove the excess of H_2S . The precipitated HgS was removed by centrifugation and the normal analysis then carried out with or without EDTA.

Inorganic phosphate was measured by the method of Berenblum & Chain (1938), as modified by Bartley (1953).

Measurement of oxidative phosphorylation. Several methods were used to obtain coupled oxidative phosphorylation: that with medium C was based on the hexokinase method of Slater & Holton (1953), and those with media A, D and E were based on the method of Kielley & Kielley (1951), variations being concerned with the substrate used, the buffer, and the time and temperature of incubation. Inorganic phosphate was always present as potassium phosphate buffer, pH 7.4. The ratio of phosphate uptake to oxygen consumption (P/O ratio) was used as a measure of oxidative phosphorylation.

Determination of radioactivity of ^{14}C -labelled amino acids. The method of Werkheiser & Bartley (1957) was used to determine radioactivity in the mitochondrial supernatant. Samples containing perchloric acid were neutralized with 2 N-KOH before counting, and planchets were dried at 110° for 15 min. In all cases a minimum of 2500 counts was taken and duplicate measurements were made after 24 hr.

Purification of and measurement of radioactivity of ^{14}C -labelled protein. Radioactivity in the mitochondrial protein was measured by scintillation counting. The mitochondrial protein was prepared by dissolving the perchloric acid precipitate of mitochondria in 3–7 ml. of N-KOH and adding a mixture of amino acids (2–5 $\mu\text{g.}$ atoms of casein-hydrolysate N/ml. of KOH). The protein was reprecipitated with 30% (w/v) trichloroacetic acid and successive washings were carried out with acetone, ethanol-ether (3:1, v/v) and ether.

Two methods for measuring the radioactivity in the protein were used. The first was based on the method of Brown & Badman (1961), except that 2,5-diphenyloxazole (4 g./l.) in toluene alone or with the addition of 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3 g./l.) in toluene was used as scintillator. Considerable quenching was found when Hyamine 10-X [*N*-benzyl-*NN*-dimethyl-*N*-2-methyl-4-(1,1,3,3-tetramethylbutyl)phenoxyethoxyethylammonium chloride monohydrate] was used as blender. The use of 98% (v/v) ethanol as blender gave a 37% increase in counts with a 1 μC standard. To eliminate all use of Hyamine the following method was finally adopted. Purified mitochondrial protein (20–50 mg.) was dissolved in 2.5 ml. of N-KOH. Of this, 0.1 ml. was added to 0.1 ml. of 2 N-HCl, and then 4 ml. of scintillator was added, giving an opaque suspension. Clarification was achieved with 1.8 ml. of 98% ethanol.

In all cases the final solutions were adapted to the dark and counted in an Ecko N612 scintillation counter at 11000 v with a 25 v bias. A minimum of 10000 counts was taken and duplicate measurements were made after 24–72 hr. The addition of 1,4-bis-(5-phenyloxazol-2-yl)benzene gave a

Table 1. *Effect of preparing and resuspending rat-liver mitochondria in different media on amino acid production at 40° and on mitochondrial water content*

Preparations and resuspensions were carried out as described in the Materials and Methods section. Incubations were at 40° for 1 hr. The incubation mixture was 3 ml. of mitochondrial suspension, 1 ml. of 0.1 M-phosphate buffer, pH 7.4 (final volume 4 ml.). Controls were either simultaneous preparations in 0.25 M-sucrose from the same liver mince, or, in Expts. 8 and 9, simultaneous resuspensions in 0.25 M-sucrose from the same mitochondrial preparation as the 'test'. Conditions were rigidly supervised so that 'test' and control resuspensions were subjected to exactly the same treatment. Expts. 4-6 represent the average of three separate experiments. I/M refers to the ratio of mitochondrial water content (mg.) to dry wt. (mg.) and was used as an indication of mitochondrial swelling. $q_{\text{amino acid}}$ is the amino acid production in $\mu\text{mole/mg. dry wt./hr.}$

Expt. no.	Preparation medium	Resuspension medium	No. of washings	Test				Control				
				Endogenous amino acid content ($\mu\text{mole/mg. dry wt.}$)		I/M	$q_{\text{amino acid}}$		Endogenous amino acid content ($\mu\text{mole/mg. dry wt.}$)	I/M	$q_{\text{amino acid}}$	
							Aerobic	Anaerobic			Aerobic	Anaerobic
1	0.25 M-Sucrose with EDTA (1 mM)	0.25 M-Sucrose	—	0.034	—	3.87	0.051	0.038	0.032	2.84	0.090	0.070
2		with EDTA (1 mM)				—	0.100	0.101		—	0.150	—
3	0.25 M-Sucrose with MnCl_2 (1 mM)	0.25 M-Sucrose with MnCl_2 (1 mM)	—	0.045	—	2.23	0.098	0.086	0.050	2.95	0.147	0.122
4-6	0.25 M-Sucrose with succinate (1 mM)	0.25 M-Sucrose with succinate (1 mM)	—	0.044 ± 0.004	—	3.06 ± 0.23	0.069 ± 0.022	0.079 ± 0.023	0.032 ± 0.006	3.45 ± 0.64	0.100 ± 0.029	0.114 ± 0.032
7	0.25 M-Sucrose	0.9% KCl (1 mM)	1	0.044	—	—	—	0.141	0.048	—	—	0.125
8	0.25 M-Sucrose	0.9% KCl	3	0.049	—	3.51	0.126	0.124	0.040	2.85	0.095	0.070
9	0.9% KCl	0.9% KCl	—	0.052	—	5.56	0.132	0.118	0.044	1.97	—	—

slight increase in the number of counts. In all experiments a zero-time count was obtained by adding perchloric acid to the incubation medium before the addition of mitochondria. A zero-time sample without added ^{14}C -labelled amino acids was used to give the background count, which was subtracted from all the values found for the incubated samples. A $1\text{ m}\mu\text{C}$ toluene standard gave 139.2 counts/min. and the test samples contained 25–50 counts/min.

Estimation of mitochondrial dry weight and water content. These were estimated as described by Alberti & Bartley (1963), the ratio of water content to dry weight (mg./mg.) (I/M) again being used as a measure of swelling.

Accuracy of results. During this work 12 sets of duplicate determinations were performed, covering the stages from the final mitochondrial preparation to the amino acid estimation. The duplicates were randomly chosen, the choice being independent of incubation time, buffer and additions to the incubation mixture. The average of each duplicate pair was taken and the error expressed as a deviation from the average. The mean error was $\pm 1.9\%$, the biggest error being $\pm 6.4\%$ in one experiment. It was therefore considered that $q_{\text{amino acid}}$ values (amino acid production in $\mu\text{moles/mg. dry wt./hr.}$) differing by more than 10% were significantly different from each other.

RESULTS

Effect of the composition of the preparation media on amino acid production and water content at 40°

Table 1 shows the amino acid production by mitochondria prepared or resuspended in different media.

Effect of EDTA. A 32–34% decrease in amino acid production aerobically and a 46% decrease anaerobically were found when 1 mM-EDTA was present in the 0.25 M-sucrose preparation medium. The initial water content of the mitochondria was greater than in 0.25 M-sucrose alone, although the initial amino acid content was unchanged. When 0.75 mM-EDTA was present in the incubation medium with mitochondria prepared in 0.25 M-sucrose, amino acid production also decreased, but less than when the mitochondria were prepared in the medium containing EDTA.

Effect of Mn^{2+} . When mitochondria were prepared in a medium containing Mn^{2+} (1 mM), amino acid production was depressed by 33% aerobically and by 30% anaerobically. Endogenous amino acid content was also lower than in the control preparation, and the mitochondrial water content was considerably lower (I/M 2.23 compared with the control value 2.95).

Effect of succinate. When 1 mM-succinate was present in the incubation medium, amino acid production was lower both aerobically and anaerobically in all three preparations tested, although succinate in the incubation medium with mitochondria prepared in 0.25 M-sucrose has been shown to give very variable results anaerobically (Alberti & Bartley, 1963). Water content was lower in all

cases when succinate was present in the preparation medium, although the endogenous amino acid content was slightly higher than in the controls.

Effect of potassium chloride. Mitochondria, whether prepared or resuspended in 0.9% potassium chloride, were very swollen compared with the control mitochondria, and amino acid production was also greater, although the endogenous amino acid content was unaltered. After one washing with 0.9% potassium chloride amino acid production was increased by 13% anaerobically, and after three washings by 77%. The addition of potassium chloride (final concn. 47 mM) to the incubation medium of mitochondria in 0.25 M-sucrose had no effect on amino acid production.

Except when EDTA was present, amino acid production increased with increase in initial water content of mitochondria. This has previously been noted for different preparations of mitochondria in 0.25 M-sucrose (Alberti & Bartley, 1963).

Effect of freezing-and-thawing on amino acid production by rat-liver homogenates

Water, 0.9% potassium chloride and 0.25 M-sucrose were used to prepare the homogenates. Homogenates prepared in 0.25 M-sucrose and 0.9% potassium chloride gave similar $q_{\text{amino acid}}$ values aerobically and anaerobically (Table 2). After freezing-and-thawing, amino acid production was increased in both media, but the increase was larger (approx. 100%) with the 0.25 M-sucrose homogenate. The increase in amino acid production after freezing-and-thawing is probably partly due to mitochondrial disruption (Alberti & Bartley, 1963). As mitochondria prepared in 0.9% potassium chloride were more swollen than when prepared in 0.25 M-sucrose (Table 1), less stimulation of amino acid production would be expected after freezing-and-thawing, but the effect of this on the total homogenate would not be large.

Homogenates prepared in water gave the same $q_{\text{amino acid}}$ values as sucrose homogenates that had been frozen-and-thawed. This was expected as suspension of particles in water causes their osmotic disruption. Freezing-and-thawing of water homogenates lowered their amino acid production by 20%, this being due possibly to excessive disruption or to denaturation of the proteinases.

The endogenous amino acid contents of the normal and frozen-and-thawed homogenates showed no significant difference.

Effect of cations on amino acid production by rat-liver mitochondria

In tris-hydrochloric acid buffer. Waley & van Heyningen (1962) investigated the effect of several

bivalent cations on proteolysis in the calf lens. Table 3 shows the effect of several of these cations on amino acid production by rat-liver mitochondria

when buffered at pH 7.4 with tris-hydrochloric acid buffer, pH 7.4 (final concn. 25 mM). Sodium chloride was added to the controls to make them

Table 2. *Amino acid production by rat-liver homogenates in various media before and after freezing-and-thawing*

In each case rat liver was homogenized six times with the appropriate medium in the Potter-Elvehjem homogenizer. Half of each homogenate was then frozen-and-thawed seven times in liquid air. All fractions were then diluted with 1 vol. of the appropriate medium. Incubation conditions and the incubation mixture were as described in Table 1.

Homogenization medium	Subsequent treatment	Amino acid content before incubation (μ mole/mg. dry wt.)	$Q_{\text{amino acid}}$		Dry wt. of incubated homogenate (mg.)
			Aerobic	Anaerobic	
0.25 M-Sucrose	None	0.257	0.042	0.069	44.9
	Freezing-and-thawing	0.251	0.118	0.140	44.0
0.9% KCl	None	0.284	0.047	0.062	44.4
	Freezing-and-thawing	0.289	0.085	0.108	41.4
Water	None	0.289	0.123	0.144	48.0
	Freezing-and-thawing	0.291	0.092	0.121	48.0

Table 3. *Effect of cations on amino acid production by rat-liver mitochondria, aerobically and anaerobically, in the presence of tris-hydrochloric acid buffer*

The incubation mixture was 2.5 ml. of mitochondrial suspension in 0.25 M-sucrose, 1 ml. of 0.1 M-tris-HCl buffer, pH 7.4, and the addition in 0.5 ml. of water. For 10 mM (final concn.) solutions of cation, NaCl controls of equivalent strength were used to allow for the ionic effect (Alberti & Bartley, 1963). Incubation conditions were as described in Table 1. Q_{O_2} 'total' was based on the O_2 uptake over 1 hr. and Q_{O_2} 'initial' was based on the O_2 uptake in the first 15 min. of incubation. Q_{CO_2} 'total' was based on the gas output over 1 hr. The chloride salts of all metals were used.

Cation	Final concn. (mM)	Aerobic			Anaerobic	
		Amino acid production (% of control)	Q_{O_2}		Amino acid production (% of control)	Q_{CO_2} total
			Initial	Total		
Mg ²⁺	10.0	76.7	12.0	9.6	43.9	—
	1.0	75.9	12.5	8.1	59.7	—
	0.1	100.0	9.7	5.3	97.1	—
Mn ²⁺	10.0	37.0	0.6	0.5	—	—
	1.0	38.9	1.0	0.8	—	—
	0.1	81.0	12.4	5.9	—	—
Ca ²⁺	10.0	78.5	0.8	0.7	—	—
	1.0	75.1	3.3	1.3	—	—
	0.1	84.2	0.5	1.2	—	—
Zn ²⁺	10.0	8.9	1.4	0.9	9.9	—
	1.0	13.0	0.0	0.0	17.5	—
	0.1	51.8	0.0	0.3	31.2	—
Hg ²⁺	0.01	89.8	—	2.8	68.2	0.8
	10.0	7.2	0.6	1.4	4.4	—
	1.0	13.6	0.0	0.0	18.6	0.5
	0.1	52.6	1.0	0.9	79.8	—
Fe ²⁺	0.01	69.3	8.8	4.4	103.6	—
	10.0	56.5	12.3	4.0	—	—
	1.0	87.8	10.9	4.9	—	—
Fe ³⁺	10.0	23.2	21.6	18.2	—	—
	1.0	97.8	9.4	3.8	—	—
Co ²⁺	10.0	22.9	—	0.8	18.8	0.0
	1.0	25.1	—	0.7	27.1	0.6
	0.1	68.5	—	1.8	67.5	0.2
None (control)	—	100.0	10.4	4.5	100.0	3.9
			± 0.3 (3) ± 0.6 (4)			

iso-osmolar, since Alberti & Bartley (1963) showed that amino acid production was inhibited by high solute concentrations.

All cations used acted as inhibitors, Hg^{2+} and Zn^{2+} being the most effective. At low concentrations (0.01 mM) Hg^{2+} had a greater inhibitory effect than Zn^{2+} anaerobically (31% inhibition compared with 10%), but the converse was true aerobically (0% compared with 32%).

It was found that Co^{2+} inhibited amino acid production strongly at 10 and 1 mM and still gave a 30% inhibition at 0.1 mM; Hg^{2+} , Zn^{2+} and Co^{2+} also inhibited respiration more than the other cations tested.

All the other cations used inhibited amino acid production at 10 mM (final concn.), and all but Fe^{3+} at 1 mM. Thus Mn^{2+} , Mg^{2+} and Ca^{2+} all inhibited amino acid production, although Penn (1961) had found that they had no effect on the proteolysis of albumin by mitochondria over the range 0.01–1.0 mM. In addition, Waley & van Heyningen (1962) found that these ions stimulated calf-lens proteinase.

In potassium phosphate buffer. When potassium phosphate buffer, pH 7.4 (final concn. 25 mM), was substituted for tris-hydrochloric acid buffer, the inhibitory effect of Mg^{2+} (1 mM) disappeared, that

of Mn^{2+} (1 mM) was much decreased, and that of Ca^{2+} was unchanged (25% inhibition at 5 mM). At these concentrations of Mn^{2+} and phosphate ions insoluble manganous phosphate is precipitated, so that the actual concentration of Mn^{2+} would be less than 1 mM.

Succinate and Mn^{2+} , when present together aerobically, show an additive inhibition of amino acid production (Table 4). Thus succinate on its own caused a 24% inhibition, Mn^{2+} alone caused an 18% inhibition, and together they caused a 41% inhibition.

At 25° (Table 4) Mg^{2+} in the presence of succinate caused a 100% stimulation of amino acid production, whereas succinate alone had a variable effect. Respiration was also increased with Mg^{2+} present. However, α -oxoglutarate caused a stimulation of amino acid production that was diminished by Mg^{2+} .

Effect of the inorganic-anion composition of the medium on amino acid production by rat-liver mitochondria

Azide, borate, carbonate, chloride, fluoride, metaphosphate, nitrate, nitrite and sulphate (final concn. 1 mM) had no effect in tris-hydrochloric acid

Table 4. *Effect of Mg^{2+} and Mn^{2+} , in the presence of substrate, on amino acid production by rat-liver mitochondria in potassium phosphate buffer*

Incubations were for 1 hr. The incubation mixture was 2.5 ml. of mitochondrial suspension in 0.25 M-sucrose, 1 ml. of 0.1 M-potassium phosphate buffer, pH 7.4, and additions, if any, in 0.5 ml. of water (final volume 4 ml.). Q_{O_2} and Q_{CO_2} values were calculated as described in Table 3.

Incubation time (min.)	Cation	Final concn. (mM)	Substrate	Final concn. (mM)	Aerobic		Anaerobic	
					$q_{\text{amino acid}}$	Q_{O_2} total	$q_{\text{amino acid}}$	Q_{CO_2} total
(a) At 40°								
60	Mn ²⁺	2	Succinate	31.2	0.068	11.1	0.073	0.4
	Mn ²⁺	2	None	—	0.096	0.9	0.103	1.0
	None	—	Succinate	31.2	0.088	14.0	0.068	0.6
	None	—	None	—	0.116	2.6	0.111	0.5
(b) At 25°								
15	Mg ²⁺	5	Succinate	12.5	0.026	27.6	—	—
	None	—	Succinate	12.5	0.009	23.6	—	—
	None	—	None	—	0.014	0.0	—	—
30	Mg ²⁺	5	Succinate	12.5	0.030	27.1	—	—
	None	—	Succinate	12.5	0.028	24.9	—	—
	None	—	None	—	0.015	1.7	—	—
15	Mg ²⁺	5	α -Oxogluta- rate	12.5	0.030	13.0	—	—
	None	—	α -Oxogluta- rate	12.5	0.065	7.5	—	—
	None	—	None	—	0.010	—	—	—
30	Mg ²⁺	5	α -Oxogluta- rate	12.5	0.011	12.7	—	—
	None	—	α -Oxogluta- rate	12.5	0.049	—	—	—
	None	—	None	—	0.014	0.9	—	—

buffer. In addition chloride at final concentrations in the range 0.28–20 mM with phosphate buffer had no effect.

In tris-hydrochloric acid buffer, only arsenate and pyrophosphate had any effect aerobically and only arsenate anaerobically. However, the effect with arsenate was small, inhibition of production being only 21% aerobically at 10 mM (final concn.), and 13%, aerobically and anaerobically, at 1 mM. Pyrophosphate (1 mM) inhibited amino acid production by 40% aerobically.

In phosphate buffer aerobically pyrophosphate (1 mM) had no effect, but at 10 mM a 67% inhibition of amino acid production was found.

Effect of various inhibitors on amino acid production by whole and broken rat-liver mitochondria

Broken, as well as whole, mitochondria were used to ensure that the inhibitors could reach intra-mitochondrial proteinases. If inhibitors showed a larger effect with whole than with broken mitochondria this would imply that there was either a structural component in the inhibitory pathway or that a membrane effect was necessary for inhibitor action.

p-Chloromercuribenzenesulphonate. *p*-Chloromercuribenzenesulphonate is an inhibitor of enzymes containing active thiol groups. In tris-hydrochloric acid buffer it (final concn. 1 mM) strongly

inhibited amino acid production by both whole and broken mitochondria, aerobically and anaerobically (70–80% inhibition) (Table 5). At 0.1 mM *p*-chloromercuribenzenesulphonate only inhibited production by whole mitochondria anaerobically. At 0.011 mM in phosphate buffer it had no effect on amino acid production.

Iodosobenzoate. This inhibitor was used by Waley & van Heyningen (1962) to inhibit calf-lens proteinase. It acts by oxidation of thiol groups. At 1 mM (final concn.) it was strongly inhibitory to amino acid production in tris-hydrochloric acid buffer (Table 5). An isolated observation in the presence of phosphate buffer showed it to be just as effective an inhibitor of proteolysis as in tris-hydrochloric acid buffer (Table 5).

Soya-bean trypsin inhibitor. In tris-hydrochloric acid buffer an inhibition of amino acid production of 29% was found aerobically (Table 5), but inhibition was much diminished with broken mitochondria.

Di-isopropyl phosphorofluoridate and EDTA. Both of these inhibitors were used over a wide concentration range at pH 7.4 in phosphate buffer (Table 5). Di-isopropyl phosphorofluoridate inhibited amino acid production by 9% at 0.002 mM (final concn.) but at 2 mM the inhibition was only 37%. Similarly, EDTA inhibited by 20% at 0.1 mM, but by only 26% at 10 mM. Both these results suggest that there is one proteolytic component sensitive to the

Table 5. *Effect of inhibitors on amino acid production by whole and broken rat-liver mitochondria in tris-hydrochloric acid and potassium phosphate buffer*

Incubation medium with tris-HCl buffer and the incubation conditions were as described in Table 3. The incubation medium with phosphate buffer was 2.5 ml. of mitochondrial suspension in 0.25 M-sucrose, 1 ml. of 0.1 M-phosphate buffer, pH 7.4, and the additions, if any, in 0.5 ml. of water (final volume 4 ml.).

Inhibitor		Amino acid production (% of control)				
		Final concn. (mM)	Whole mitochondria		Broken mitochondria	
			Aerobic	Anaerobic	Aerobic	Anaerobic
(a) In phosphate buffer						
Borohydride	1.0	128	127	—	—	
EDTA	10	74	—	—	—	
	0.1	80	—	—	—	
	0.75	—	64	—	—	
Di-isopropyl phosphorofluoridate	2.0	63	—	—	—	
	0.02	79	—	—	—	
	0.0002	93	—	—	—	
	0.1	—	86	—	—	
Iodosobenzoate	10	31	—	—	—	
(b) In tris-HCl buffer						
Iodosobenzoate	1.0	31	22	27	21	
	0.1	94	92	—	76	
p-Chloromercuribenzenesulphonate	1.0	25	25	31	32	
	0.1	95	74	—	90	
Soya-bean trypsin inhibitor	0.25 mg./ml.	71	93	98	87	

particular inhibitor and that rat-liver mitochondria contain a mixture of proteinases active at neutral pH.

In an experiment at pH 5.8 with phosphate buffer amino acid production was increased by 59% in the presence of di-isopropyl phosphorofluoridate (0.1 mM); this is further evidence for the multiplicity of mitochondrial proteolytic enzymes.

Other inhibitors. Potassium borohydride, sodium dithionite (both at final concn. 0.001–1.0 mM), and 2,4-dinitrophenol (0.1 mM) had no effect on amino acid production in tris-hydrochloric acid buffer. However, borohydride stimulated production by 28% in phosphate buffer, the other two inhibitors having no effect. Similarly, antimycin A (0.001 mM), sodium Amytal (1 mM) and sodium cyanide (10 mM) had no effect in phosphate buffer at pH 7.4. In the presence of substrate cyanide had no effect but 2,4-dinitrophenol tended to diminish the inhibitory action of succinate and pyruvate (Alberti & Bartley, 1963).

Effect of various inhibitors on amino acid production by rat-liver homogenate and subcellular fractions

The pattern of inhibition produced by the six inhibitors (borohydride, di-isopropyl phosphorofluoridate, soya-bean trypsin inhibitor, EDTA, iodosobenzoate, *p*-chloromercuribenzenesulphonate) is individual to each cell fraction (Fig. 1).

Amino acid production by the nuclear fraction was decreased by all the inhibitors used, ranging from a 76% inhibition with *p*-chloromercuribenzenesulphonate to a 21% inhibition with borohydride. The latter had no effect on the other fractions and the homogenate was inhibited by only 10%, this probably being due to its content of nuclear proteinases.

Amino acid production by the final supernatant (cell sap) was least affected by the inhibitors used, the biggest inhibition being 33% by *p*-chloromercuribenzenesulphonate.

The pattern of inhibition found for the 'fluffy layer' was similar to that of the final supernatant except that iodosobenzoate inhibited production by 65% compared with 26%. The fluffy-layer pattern differed greatly from those found for the mitochondrial and microsomal fractions. This lends weight to the hypothesis that fluffy layer contains some unique components and is not just a mixture of other fractions (see also Alberti & Bartley, 1963).

The effects of the inhibitors on the homogenate should indicate whether there is any interaction between fractions. Inhibition of the homogenate was greater than with any individual fraction only with di-isopropyl phosphorofluoridate, and less only with soya-bean trypsin inhibitor, so that interaction is definitely proved only with these two inhibitors.

The general pattern of inhibition suggests that

Fig. 1. Effect of inhibitors on the production of amino acids by rat-liver homogenate and cell fractions. All fractions were incubated for 1 hr. at 40° aerobically. The incubation medium contained 2.5 ml. of fraction in 0.25 M-sucrose, 1 ml. of 0.1 M-tris-HCl buffer, pH 7.4, and 0.5 ml. of inhibitor in water (final volume 4 ml.). Amino acid production is given as a percentage of the control value without inhibitor. The inhibitors were all 1 mM (final concn.), except for soya-bean trypsin inhibitor, which was present at a concentration of 0.5 mg./ml. of incubation medium. Fractions: (a) whole homogenate; (b) nuclei; (c) mitochondria; (d) fluffy layer; (e) microsomes; (f) final supernatant. Inhibitors: ■, potassium borohydride; □, di-isopropyl phosphorofluoridate; ▨, soya-bean trypsin inhibitor; ▤, EDTA; ▥, iodosobenzoate; ▦, *p*-chloromercuribenzenesulphonate.

Table 6. *Effect of oxidizable substrate on the production of amino acids by rat-liver mitochondria*

The incubation mixture was 2.5 ml. of mitochondrial suspension in 0.25 M-sucrose, 1 ml. of 0.1 M-buffer (either tris-HCl or potassium phosphate), pH 7.4, and 0.5 ml. of substrate in water. Incubation conditions were as described in Table 3. Amino acid production is expressed as a percentage of that in the control incubation without substrate. Results indicated by an asterisk (*) incorporate those obtained by Alberti & Bartley (1963).

Substrate	Final concn. (mm)	Tris-HCl buffer		Phosphate buffer	
		Amino acid production (% of control)	No. of expts.	Amino acid production (% of control)	No. of expts.
Succinate	10-12.5	68	1	77 (± 4)*	3
α -Oxoglutarate	10-12.5	66 (± 26)	5	75 (± 20)*	6
Fumarate	10	68	1	86	1
Malate	10	72	1	87	1
Isocitrate	10	52	1	76	1
Pyruvate	10	80	1	72 (± 4)*	2
α -Glycerophosphate	10	83	1	98	1
Palmitate	1	84	1	102	1
Octanoate	1	74 (± 20)	3	93 (± 5)	2
	10	—	—	76	1
Octanoate + malate	10	—	—	71	1

Table 7. *Effect of CoA, in the presence or absence of α -oxoglutarate, AMP and NAD on the production of amino acids by rat-liver mitochondria*

Incubation conditions and medium were as described in Table 3.

Addition to incubation medium	Final concn.	Amino acid production (% of control)			
		Tris-HCl buffer		Phosphate buffer	
		Aerobic	Anaerobic	Aerobic	Anaerobic
CoA	1 mg./ml.	129	141	95	65
CoA + α -oxoglutarate	1 mg./ml.	85	98	110	59
α -oxoglutarate	12.5 mm				
α -Oxoglutarate	12.5 mm	80	133	48	48

the proteolytic activity of each fraction is due to a mixture of proteolytic enzymes.

Effect of oxidizable substrate on amino acid production by rat-liver mitochondria aerobically

Without cofactors. Some decrease in amino acid production together with a stimulation of respiration was found with all substrates tested in phosphate buffer, except α -glycerophosphate and palmitate. Inhibition of amino acid production was greater in tris-hydrochloric acid than in phosphate buffer (Table 6). Q_{O_2} values were also greater in tris-hydrochloric acid than in phosphate buffer.

With cofactors. Penn (1961) reported that CoA was essential for the breakdown of endogenous mitochondrial protein, and that NAD had a similar effect but to a smaller extent (10%).

Table 7 shows that in tris-hydrochloric acid

buffer CoA stimulated amino acid production both aerobically and anaerobically, confirming part of Penn's (1961) results. On the addition of α -oxoglutarate anaerobically the stimulation of production was abolished, but aerobically a decrease in amino acid production was found (15%) similar to that obtained with oxoglutarate alone (20%). In phosphate buffer CoA had no effect aerobically and inhibited amino acid production by 35% anaerobically. This is in accord with Penn's (1961) view that phosphate abolishes the CoA effect. However, Penn (1961) found little endogenous proteolytic activity in the absence of CoA, whereas we have always found a large production of amino acids, independent of added CoA.

NAD (final concn. 1 mm) aerobically in tris-hydrochloric acid caused a 36% stimulation of amino acid production, but AMP (1-5.5 mm) on its own had no effect on either tris-hydrochloric acid

or phosphate buffer. The NAD effect was abolished by the addition of succinate.

Effect of added amino acids and GSH on amino acid production by whole and broken mitochondria

Glutamate was shown to have no effect on net amino acid production (Alberti & Bartley, 1963) in phosphate buffer. In tris-hydrochloric acid buffer, cysteine (1 mM), which is known to activate many proteinases (Greenbaum & Sherman, 1962; Kuzovleva & Chung-yen, 1959), and GSH (1 mM) both stimulated amino acid production by whole mitochondria, by 29% aerobically and by 10–13% anaerobically. GSH (1 mM) also caused a 29% stimulation of amino acid production by broken mitochondria, but cysteine (1 mM) had no effect.

GSH may act as a substrate. Cysteine affected only whole mitochondria and probably acted as a swelling agent, although all the effects were small.

Glycine had no effect at the concentrations tested (0.1 and 1 mM).

Effect of hormones on amino acid production by rat-liver mitochondria in phosphate buffer aerobically

Several hormones have been shown to influence amino acid incorporation into mitochondrial protein *in vivo* (Korner, 1960a,b), and thyroxine has been shown to stimulate protein breakdown *in vivo* (Scow, 1954). Also, several hormones cause mitochondrial swelling. Table 8 shows their effects on amino acid production.

Adrenocorticotrophic hormone and growth hormone both stimulated amino acid production by 20% or more. The proviso has to be made that they may have acted as substrates.

Thyroxine, despite its known effect on mitochondrial swelling, and cortisone had no effect on amino acid production. Insulin, however, caused a 25% inhibition of production, despite its being a protein

and its action as a swelling agent (Lehninger & Neubert, 1961).

Relation of oxidative phosphorylation to amino acid production by rat-liver mitochondria at 25° and 40°

The incorporation of amino acids into the protein of rat-liver mitochondria is energy-dependent (McLean, Cohn, Brandt & Simpson, 1958), as is the reversal of mitochondrial swelling (Macfarlane & Spencer, 1953). In terms of absolute amounts amino acid incorporation is negligible compared with amino acid production. However, reversal of mitochondrial swelling should be sufficient for it to be assumed that amino acid production would be inhibited during oxidative phosphorylation.

At 25°. Table 9 shows that at 25°, during oxidative phosphorylation, amino acid production was stimulated, the increase in $q_{\text{amino acid}}$ ranging from 0.003 to 0.035 (0.062 at 30°), regardless of medium, substrate and incubation time.

The use of hexokinase (medium C) was abandoned because the hexokinase preparation contained free amino acids. The P/O ratios showed great variability but in all cases some phosphorylation took place.

At 40°. At this temperature with medium D amino acid production was stimulated by 72% (Table 10). It was possible that, as phosphate buffer was acting as the source of inorganic phosphate, the stimulation of amino acid production was partly due to a fall in the solute concentration (Alberti & Bartley, 1963). Medium E therefore incorporated a high concentration of tris-hydrochloric acid buffer (final concn. 0.125 M), as well as phosphate. In the two experiments carried out with this medium, stimulation of amino acid production was 27 and 154%. In all cases the P/O ratio was in the same range (0.94–1.10).

Table 10 shows that the addition of 2,4-dinitrophenol to the phosphorylating medium both de-

Table 8. *Effect of hormones on the aerobic production of amino acids by rat-liver mitochondria in phosphate buffer*

Incubation conditions and mixture were as described in Table 3. Blanks of hormones were incubated without mitochondria and the ninhydrin-positive value obtained was deducted from the value found with mitochondria present. Q_{O_2} values were calculated as described in Table 3.

Hormone	Final concn. (mM)	Amino acid production (% of control)	Q_{O_2}	
			Initial	Total
Adrenocorticotrophic hormone	0.01	124.0	5.5	4.0
Growth hormone	0.01	121.3	3.2	2.8
Cortisone acetate	0.01	108.0	6.2	4.4
Thyroxine	0.01	97.3	5.0	3.9
Insulin	0.01	72.3	4.3	3.4
None (control)	—	100.0	4.6	3.4

Table 9. *Amino acid production by rat-liver mitochondria during oxidative phosphorylation at 25° in various media*

Medium A contained: MgCl₂ (final concn. 10 mm), AMP (20 mm), potassium phosphate buffer, pH 7.4 (25 mm), substrate (12.5 mm) and sucrose (125 mm) in a final volume of 4 ml. Medium B contained: MgCl₂ (7.9 mm), ADP (15.8 mm), phosphate buffer, pH 7.4 (26.3 mm), substrate (10.5 mm) and sucrose (132 mm) in a final volume of 3.9 ml. Medium C contained: MgCl₂ (7.3 mm), ATP (0.73 mm), phosphate buffer, pH 7.4 (24.4 mm), succinate (12.2 mm), sucrose (122 mm), glucose (12.2 mm) and hexokinase (0.49 mg./ml. of incubation medium) in a final volume of 4.1 ml. Controls were 3 ml. of mitochondrial suspension in 0.25 M-sucrose and 1 ml. of 0.1 M-potassium phosphate buffer, pH 7.4. All incubations were done in air and at 25°, apart from the experiment marked with an asterisk (*), which was done at 30°.

Medium	Incubation time (min.)	Substrate	$q_{\text{amino acid}}$	P/O ratio	Q_{O_2} total
A	30	α -Oxoglutarate	0.037	—	—
Control	30	None	0.005	—	—
A	30	Succinate	0.018	2.03	—
Control	30	None	0.015	0.00	—
B	10	Succinate	0.035	0.98	42.7
Control	10	None	0.000	0.00	—
B	20	Succinate	0.046	0.54	43.8
Control	20	None	0.023	0.00	—
B	10	Succinate*	0.106	1.71	60.2
Control	10	None	0.044	0.00	—
C	30	Succinate	0.034	1.82	56.4
Control	30	None	0.024	0.00	2.0
C	60	Succinate	0.047	1.87	43.8
Control	60	None	0.013	0.00	1.5
Control + hexokinase	60	None	0.013	0.00	2.3

Table 10. *Amino acid production by rat-liver mitochondria in phosphorylating media at 40°, with and without 2,4-dinitrophenol*

Incubations were at 40° for 35 min. in air. Medium D contained: mitochondria in sucrose (final concn. 125 mm), phosphate buffer, pH 7.4 (25 mm), succinate (12.5 mm), AMP (10 mm) and MgCl₂ (5 mm) in a final volume of 4 ml. When present, 2,4-dinitrophenol was 0.1 mm. Medium E contained: mitochondria in sucrose (125 mm), tris-HCl buffer, pH 7.4 (125 mm), phosphate buffer, pH 7.4 (12.5 mm), succinate (12.5 mm), AMP (10 mm) and MgCl₂ (5 mm) in a final volume of 4 ml. When present, 2,4-dinitrophenol was 0.002 mm. The controls consisted of 2 ml. of mitochondria in 0.25 M-sucrose, 1 ml. of water and 1 ml. of appropriate buffer mixture.

Expt.	Medium	Amino acid production (% of control)	P/O ratio	Q_{O_2} total
1	D	172	0.94	58.00
	D + 2,4-dinitrophenol	92	0.00	53.80
	Control	100	0.00	0.59
2	E	127	1.10	63.71
	E + 2,4-dinitrophenol	110	0.00	41.34
3	E	254	1.08	50.58
	E + 2,4-dinitrophenol	105	0.00	34.28
	E + 2,4-dinitrophenol - MgCl ₂ - AMP	105	0.02	39.09
	E - MgCl ₂ - AMP	100	0.09	37.37

creased amino acid production to approximately control levels and completely uncoupled oxidation from phosphorylation.

Relation between amino acid incorporation and amino acid production in rat-liver mitochondria

Roodyn, Reis & Work (1961) showed that amino acids from ¹⁴C-labelled algal-protein hydrolysate

could be incorporated into rat-liver mitochondria protein. Tests under the conditions of these workers (Table 11) show that without added amino acids there was the usual increase in amino acids on incubation. As no buffer was present apart from phosphate the 51% increase in amino acid production, when phosphate was omitted, was probably due to a fall in pH (the pH of unbuffered mitochondria being about 6.8), particularly as the stimulation

Table 11. *Amino acid production by rat-liver mitochondria in an amino acid incorporation medium, and the effect of omission of some of the constituents*

The medium was based on 'medium B' of Roodyn *et al.* (1961). The complete system contained: sucrose (final concn. 100 mM), phosphate buffer, pH 7.4 (15 mM), AMP (4 mM), succinate (10 mM), NAD (0.5 mM), nicotinamide (20 mM), EDTA (1.25 mM) and KCl (40 mM). Tris-HCl buffer, pH 7.4, was 15 mM (*) or 100 mM (†). The total volume for incubation including mitochondria was 5 ml. With 'phosphate only' the medium comprised 3 ml. of mitochondrial suspension in 0.25 M-sucrose, 1 ml. of 0.1 M-phosphate buffer, pH 7.4, and 1 ml. of water. The water was replaced by 1 ml. of 0.5 M-tris-HCl buffer, pH 7.4, for 'phosphate + tris-HCl† only'. Incubations were at 37° for 1 hr.

Medium	Amino acid production (% of complete system)	P/O ratio	Q_{O_2} total	Phosphate consumed (μ moles)
Complete system	100.0	0.70	22.4	29.0
Complete system—AMP	88.0	0.03	21.4	0.1
Complete system—succinate	67.5	0.00	2.7	5.2
Complete system—phosphate	150.5	—	21.8	—
Complete system—KCl	102.0	0.63	23.4	24.4
Complete system—nicotinamide	101.5	0.68	24.0	27.0
Complete system—NAD	78.5	0.71	22.1	26.0
Complete system—EDTA	87.0	0.78	25.2	28.2
Complete system—phosphate + tris-HCl†	91.0	—	17.6	—
Complete system + tris-HCl*	90.0	0.81	21.6	26.2
Phosphate only	92.5	—	0.9	—
Phosphate + tris-HCl† only	115.5	—	0.8	—

Table 12. *Amino acid production and 14 C-labelled amino acid incorporation by rat-liver mitochondria, with and without chloramphenicol*

The incubation mixture was the complete system described in Table 3 plus tris-HCl buffer, pH 7.4 (10 mM), and 0.5 μ C of 14 C-labelled algal-protein hydrolysate (0.0375 mg. of casein hydrolysate/ml. of medium). Chloramphenicol, when present, was 0.25 mM (final concn.). Incubations were at 37° in air. Q_{O_2} total was calculated as described in Table 3.

Medium	Incubation time (min.)	Amino acid incorporation (μ mo/g. dry wt.)	Amino acid production (μ mole/mg. dry wt.)	P/O ratio	Q_{O_2} total
Complete system	30	110.1	0.169	0.8	36.5
Complete system	60	196.0	0.257	0.3	44.3
Complete system—added amino acids	60	0.0	0.215	0.4	42.0
Complete system—chloramphenicol	60	9.4	0.219	0.4	35.2

was eliminated by replacing phosphate with tris-hydrochloric acid buffer.

Removal of AMP and succinate from the medium gave decreases in amino acid production of 12 and 33% respectively. In both cases there was no net phosphorylation. Removal of NAD decreased amino acid production by 22%, confirmation of the observation (see above) that NAD increased amino acid production. Most of the stimulatory effects of removal of constituents from the medium could be due to lowering the solute concentration of the medium. Comparison with results obtained from incubating mitochondria in phosphate and sucrose alone is difficult owing to the large difference in osmotic constitution of the two media.

Table 12 shows the results with added 14 C-labelled amino acids. The highest incorporations were shown in the samples with the highest amino acid productions. Thus after incubation for 1 hr. in the complete system 196 μ C was incorporated/g. of mitochondrial protein and 0.257 μ mole of amino acid produced/mg., whereas in the presence of chloramphenicol the equivalent values were 9 μ C/g. and 0.209 μ mole/mg. Chloramphenicol decreased amino acid production to the level of the sample incubated without added amino acids, implying that actual incorporation must be occurring for maximal amino acid liberation. Chloramphenicol had no effect on oxidative phosphorylation, so it could not inhibit by this mechanism.

Production of amino acids by rat-liver particles in the presence of added protein

Three types of protein have been used: egg albumin, denatured mitochondrial protein and ^{14}C -labelled algal protein.

Albumin. Penn (1960, 1961) established the conditions under which mitochondria will break down [^{14}C]albumin. In phosphate buffer in the presence of 2mg. of albumin/ml. the ninhydrin-positive products found after the incubation of whole mitochondria were increased by 35% in the presence of albumin aerobically, but slightly decreased anaerobically (Table 13).

The insoluble fraction of broken mitochondria has a low $q_{\text{amino acid}}$ in the presence of buffer alone. In the presence of albumin anaerobically the $q_{\text{amino acid}}$ was increased by 28%. With the soluble fraction the $q_{\text{amino acid}}$ was increased from 0.120 to 0.510 when albumin was present.

Denatured mitochondrial protein. Mitochondrial protein, denatured by boiling and added to normal mitochondria, gave no stimulation of amino acid production, either aerobically or anaerobically at 40°.

^{14}C -labelled algal protein. In the presence of ^{14}C -labelled algal protein there was only a slight increase in amino acid production in either tris-hydrochloric acid or phosphate buffer on incubation of whole mitochondria. The maximum increase (in tris-hydrochloric acid buffer aerobically) represented 2.3% of the added protein and 5% of the total amino acid production.

Table 13. *Amino acid production by whole, and subfractions of broken, rat-liver mitochondria in the presence of egg albumin*

The incubation medium comprised 3 ml. of mitochondrial suspension or fraction in 0.25M-sucrose, with or without albumin (final concn. 2 mg./ml.), and 1 ml. of 0.1M-potassium phosphate buffer, pH 7.4. Incubations were for 1 hr. for whole mitochondria and 30 min. for fractions. A blank without mitochondria was incubated with albumin. For the fractionation broken mitochondria were centrifuged for 39 000g-min., the precipitate being the insoluble fraction, the supernatant the soluble fraction.

Fraction	Albumin (mg./ml.)	$q_{\text{amino acid}}$	
		Aerobic	Anaerobic
Whole mitochondria	0	0.135	0.193
	2	0.182	0.171
Insoluble fraction	0	—	0.019
	2	—	0.025
Soluble fraction	0	—	0.120
	2	—	0.510

DISCUSSION

Characteristics of the proteolytic reaction in rat-liver mitochondria at pH 7.4. In a previous paper (Alberti & Bartley, 1963) we showed that isolated rat-liver mitochondria produced amino acids when incubated at 40° (approx. 0.150 $\mu\text{mole/mg. dry wt./hr.}$); the rate of production varied inversely with solute concentration, and it was lower in phosphate buffer than in tris-hydrochloric acid buffer. Amino acid production was lowered aerobically in the presence of certain oxidizable substrates, whereas disrupting the mitochondria raised amino acid production by 50–100%. The present work has further described the factors modifying that rate at which the amino acids are liberated.

Amino acid production occurs irrespective of the preparation medium. The water content of the mitochondria at the end of the preparation procedure is often an indication of the subsequent rate of amino acid production (Alberti & Bartley, 1963). Thus mitochondria prepared in potassium chloride solution were swollen and amino acid production was increased, whereas in the sucrose- Mn^{2+} medium the water content and amino acid production of the mitochondria were both lowered.

All the cations tested lowered amino acid production, but the mechanisms of inhibition may differ. Thus Mn^{2+} is known to cause mitochondrial shrinking (Amoore & Bartley, 1958), as do Mg^{2+} and Co^{2+} (Tapley, 1956); Co^{2+} also depresses oxidation in rat-liver mitochondria (Dingle, Heath, Webb & Daniel, 1962); Fe^{3+} stimulates oxidation, and Hg^{2+} attacks thiol groups.

Pyrophosphate and arsenate inhibited amino acid production, other anions, including fluoride (a potent inhibitor of a neutral rat-liver esterase; Hanson & Kleine, 1959), being ineffective.

The reaction in whole and broken mitochondria was strongly inhibited by *p*-chloromercuribenzene-sulphonate (1mM) and iodosobenzoate (1mM), suggesting that a thiol group may be at or near the active centre of one of the proteinases. Waley & van Heyningen (1962) suggested that iodosobenzoate inhibits lens proteinases far more than peptidases. An inhibition of only 30% was found with diisopropyl phosphorofluoridate (10mM), which attacks active centres containing serine (Hartley, 1960), suggesting that most of the enzymes involved do not belong to this category.

EDTA (10mM) gave a 25% inhibition of amino acid production. EDTA also increased endogenous respiration, which could lower amino acid production, although Kunz (1960) has reported that EDTA inhibits the oxidation of succinate by rat-liver mitochondria. EDTA also inhibits most types of mitochondrial swelling (Hunter *et al.* 1959). Soya-bean trypsin inhibitor diminished amino acid

production by 29%. Cathepsins B and C, which have been localized in the rat-liver mitochondrial fraction, are unaffected by this inhibitor (Finkenstaedt, 1957).

NAD stimulated amino acid production aerobically, substrate reversing the effect. CoA in tris-hydrochloric acid buffer also stimulated amino acid production, the presence of substrate or phosphate preventing the CoA stimulation.

Cysteine and GSH both increased amino acid production. Certain cathepsins (Rademaker, 1959) are activated by cysteine, but we found that it had no effect with broken mitochondria. The implication is that it stimulated production through its action as a swelling agent. GSH, on the other hand, although a swelling agent, stimulated production by both whole and broken mitochondria, and may have acted as a substrate.

Growth hormone and adrenocorticotrophic hormone stimulated the production of amino acids. They may have acted as substrates. There are two other possible mechanisms for growth hormone: as a swelling agent or by increasing the NAD/NADH₂ ratio. Both effects have been shown by Melhuish & Greenbaum (1961). Insulin inhibited amino acid production, despite its protein nature and its action as a swelling agent (Lehninger & Neubert, 1961). Manchester (1961) reported that insulin decreased amino acid production by rat diaphragm, and suggested that it acted by increasing amino acid incorporation. However, stimulation of amino acid incorporation into mitochondrial protein by insulin has not yet been shown *in vitro*.

Amino acid production was stimulated in the presence of coupled oxidative phosphorylation, the stimulation being prevented by 2,4-dinitrophenol. This suggests the presence of an energy-dependent component of the mitochondrial proteolytic system.

When labelled amino acids were added to a phosphorylating medium amino acid production was further increased. The addition of chloramphenicol inhibited both incorporation and production, oxidative phosphorylation being unaffected, suggesting that some of the mitochondrial proteolytic system is directly linked with the incorporation system.

The continued production of amino acids during amino acid incorporation will make a considerable difference to calculations of the specific activity of amino acid pools (e.g. Roodyn *et al.* 1961). During 30 min. incubation (Table 12) the endogenous amino acids increased from 2.2 to 6.9 μ moles. Added amino acids amounted to 2.2 μ moles, and therefore after 30 min. incubation the specific activity of the pool fell by half. If the calculation is carried further it will be seen that amino acid incorporation will be underestimated.

Thus, if the values of Roodyn *et al.* (1961) are taken, it can be shown that they found that in the second hour of incubation radioactivity in the mitochondrial protein increased by only 27% of the value obtained for the first hour. However, if it is assumed that the size of the amino acid pool is increased three times in the first hour and five times by the end of the second hour, then their incorporation becomes a straight line function of time over the 2 hr. period.

Albumin stimulated amino acid incorporation by 35% aerobically. This could either be proteolysis of the albumin or stimulation of oxidative phosphorylation and amino acid incorporation (Truman & Korner, 1962). Other exogenous proteins were minimally attacked. van Heyningen & Waley (1963) reported a similar difficulty in finding a suitable substrate for the neutral proteinase of lens. Whereas most of the proteolytic activity towards both endogenous protein and albumin rests in the soluble fraction of broken mitochondria, most of the incorporation takes place in the sediment (Roodyn *et al.* 1961).

Hartley (1960) classified proteolytic enzymes in five groups: (a) serine proteinases; (b) thiol proteinases; (c) acid proteinases; (d) metal proteinases; (e) miscellaneous proteinases. Unfortunately the last group is large. The enzymes of group (a) contain a serine group in the active centre that reacts uniquely with organophosphorus compounds, e.g. trypsin (Naughton, Sanger, Hartley & Shaw, 1960). The thiol proteinases are all activated by thiol compounds such as cysteine and GSH, most acid cathepsins belonging to this group. Though the evidence was not strong for the existence of a neutral cysteine- or GSH-activated proteinase in mitochondria, proteolysis was strongly inhibited by *p*-chloromercuribenzenesulphonate, iodosobenzoate and Hg²⁺, all specific reagents for thiol groups. Acid proteinases (group c) are present in mitochondria but their role in neutral proteolysis is unknown. The proteinases of group (d) are activated by metal ions, mainly Co²⁺ and Mn²⁺. This effect was not found (Table 3).

Our system shows characteristics of serine and thiol proteinases, but, so long as the types of bonds attacked are unknown and the enzymes remain unisolated, further classification will be difficult, and fruitless.

Comparison with other proteolytic systems of rat-liver mitochondria. The proteolytic system described in the present work and that described by Penn (1960, 1961) for the degradation of albumin by mitochondria have certain features in common. They are both stimulated by CoA and NAD, and both inhibited by phosphate and pyrophosphate. However, Penn (1960, 1961) found no proteolysis in the absence of CoA or NAD or ATP. It is probable

that his system comprises a small part of the total mitochondrial system.

Korner & Tarver (1957) also studied the release of amino acids from labelled protein at pH 7.3 in a medium comprising sucrose (0.25M), magnesium chloride (2.5mM), manganese chloride (1mM) and mixed amino acids (1.0mg./ml.). They found that 2,4-dinitrophenol (0.1mM) inhibited amino acid release, as did the addition of ATP to the medium. We have been unable to confirm either of these results, owing probably to the differences in the respective media.

Beeken & Imredy (1962) were unable to detect any breakdown of radioiodinated serum albumin at pH 7.4 by rat-liver mitochondria, but significant amounts of radioactivity were released at pH 5.5 and 5, the latter being independent of ATP and CoA. They also used broken mitochondria at pH 7 with no result, whereas we found the supernatant of broken mitochondria to be the most active fraction. Unless there is a significant difference between the types of albumin used by Penn (1960), Beeken & Imredy (1962) and ourselves, one must assume that albumin is acting otherwise than as a substrate.

Several other proteinases have been found in the rat-liver mitochondrial fraction. Finkenstaedt (1957) found cathepsins B and C. Both have acid pH optima, as has the cathepsin A found by Rademaker (1959), but some catheptic activity still remains at pH 7.4 (Dingle, 1961), although this represents only about 3% of maximum activity. Cathepsins B and C are cysteine-dependent. Maver & Greco (1951) showed that benzoylarginine amide-splitting ability was at a maximum at pH 7.2, with some activity still at neutral pH. A carboxypeptidase has also been shown in mitochondria (Hanson, Hermann & Blech, 1959; Rademaker, 1959). Until further work is done on these enzymes their role in the proteolytic reaction at pH 7.4 cannot be evaluated.

Proteolysis in rat-liver homogenates and subcellular fractions. Rat-liver homogenates produced amino acids at a slightly lower rate in 0.9% potassium chloride than in 0.25M-sucrose. Homogenates in water gave similar $q_{\text{amino acid}}$ values to 0.25M-sucrose homogenates that had been frozen-and-thawed, i.e. twice the normal values for sucrose homogenates. Freezing-and-thawing and hypo-osmotic treatment are the accepted means for rupturing lysosomes (Gianetto & de Duve, 1955). They also rupture mitochondria. Dingle (1961) showed that lysosomal cathepsin, after hypo-osmotic treatment of the lysosomal fractions, had very little activity at pH 7, and the increase in proteolysis in the homogenate is too great to be accounted for by rupture of mitochondria alone. Therefore activation or disruption or both of some other fraction, such as nuclei, must be responsible. Because of the com-

plexity of the system the experiments on homogenates are not capable of precise interpretation. Lysosomes cannot be entirely ruled out, as Waley & van Heyningen (1962) have pointed out that the usual method for measuring catheptic activity, i.e. measuring the extinction at 280m μ , is not a particularly sensitive method of measuring peptide-bond splitting.

Several proteinases and peptidases have been localized in fractions of rat liver by using artificial substrates. Hanson & Blech (1959) reported that several di- and tri-peptidases were present in the soluble fraction, as was most of the leucinamide-splitting activity (Maver & Greco, 1951). Similarly, Rademaker & Soons (1957) found that glycylglycylglycine and glycylglycine were split primarily by the soluble fraction, and carboxypeptidase was present in the mitochondrial and microsomal fractions (Hanson *et al.* 1959).

Comparison with these findings is difficult, as perusal of the literature has revealed no work involving native substrate, and few references to the effects of inhibitors. Wilcox & Fried (1963) showed that glycylglycine dipeptidase was entirely inhibited by EDTA, being Co^{2+} - and Mn^{2+} -dependent. We found little effect of EDTA with the soluble fraction. Also, as is to be expected, the distribution of endogenous proteolytic activity matched none of the distributions of specific peptidases. All that can be said at present is that, on the basis of Hartley's (1960) classification, nuclei and microsomes as well as mitochondria contain serine proteinases and thiol proteinases, whereas fluffy layer and the supernatant have a lower content of thiol proteinases, as measured by sensitivity to *p*-chloromercuribenzenesulphonate, and little or no serine proteinase content. This presents yet further evidence that fluffy layer is a distinct fraction, and not just a mixture of mitochondria and microsomes.

It is clear that amino acid production is an important phenomenon, quantitatively significant, in all isolated fractions of rat liver under many different conditions, and that unless it is taken into account many studies on amino acid incorporation and metabolism become quantitatively inexact.

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REFERENCES

- Alberti, K. G. M. M. & Bartley, W. (1963). *Biochem. J.* **87**, 104.
- Amoore, J. E. & Bartley, W. (1958). *Biochem. J.* **69**, 223.
- Bartley, W. (1953). *Biochem. J.* **54**, 677.
- Beeken, W. L. & Imredy, K. (1962). *Biophys. Acta*, **62**, 579.

- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
- Brown, W. O. & Badman, H. G. (1961). *Biochem. J.* **78**, 571.
- Dingle, J. T. (1961). *Biochem. J.* **79**, 509.
- Dingle, J. T., Heath, J. C., Webb, M. & Daniel, M. (1962). *Biochim. biophys. Acta*, **65**, 34.
- Finkensaedt, J. T. (1957). *Proc. Soc. exp. Biol., N.Y.*, **95**, 302.
- Gianetto, R. & de Duve, C. (1955). *Biochem. J.* **59**, 433.
- Greenbaum, L. M. & Sherman, R. (1962). *J. biol. Chem.* **237**, 1082.
- Hanson, H. & Blech, W. (1959). *Hoppe-Seyl. Z.* **315**, 191.
- Hanson, H., Hermann, P. & Blech, W. (1959). *Hoppe-Seyl. Z.* **315**, 201.
- Hanson, H. & Kleine, R. (1959). *Hoppe-Seyl. Z.* **315**, 208.
- Hartley, B. S. (1960). *Annu. Rev. Biochem.* **29**, 45.
- Hunter, F. E., Levy, J. F., Fink, J., Schutz, B., Guerra, F. & Hurwitz, A. (1959). *J. biol. Chem.* **234**, 2176.
- Kielley, W. W. & Kielley, R. K. (1951). *J. biol. Chem.* **191**, 485.
- Korner, A. (1960a). *J. Endocrin.* **20**, 256.
- Korner, A. (1960b). *Biochem. J.* **74**, 462.
- Korner, A. & Tarver, H. (1957). *J. gen. Physiol.* **41**, 219.
- Kunz, W. (1960). *Naturwissenschaften*, **47**, 381.
- Kuzovleva, O. B. & Chung-yen, W. (1959). *Biokhimiya*, **24**, 550.
- Lehninger, A. L. & Neubert, D. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1929.
- Macfarlane, M. G. & Spencer, A. G. (1953). *Biochem. J.* **54**, 569.
- McLean, J. R., Cohn, G. L., Brandt, I. K. & Simpson, M. V. (1958). *J. biol. Chem.* **233**, 657.
- Manchester, K. L. (1961). *Biochem. J.* **81**, 135.
- Maver, M. E. & Greco, A. E. (1951). *J. nat. Cancer Inst.* **12**, 37.
- Melhuish, A. H. & Greenbaum, A. L. (1961). *Biochem. J.* **78**, 392.
- Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C. (1960). *Biochem. J.* **77**, 149.
- Penn, N. W. (1960). *Biochim. biophys. Acta*, **37**, 55.
- Penn, N. W. (1961). *Biochim. biophys. Acta*, **53**, 490.
- Rademaker, W. (1959). Ph.D. Thesis: University of Utrecht.
- Rademaker, W. & Soons, J. B. J. (1957). *Biochim. biophys. Acta*, **24**, 451.
- Roodyn, D. B., Reis, P. J. & Work, T. S. (1961). *Biochem. J.* **80**, 9.
- Rosen, H. (1957). *Arch. Biochem. Biophys.* **67**, 10.
- Scow, R. O. (1954). *Endocrinology*, **55**, 344.
- Slater, E. C. & Holton, F. A. (1953). *Biochem. J.* **55**, 530.
- Tapley, D. F. (1956). *J. biol. Chem.* **222**, 325.
- Truman, D. E. S. & Korner, A. (1962). *Biochem. J.* **83**, 588.
- van Heyningen, R. & Waley, S. G. (1963). *Biochem. J.* **86**, 92.
- Waley, S. G. & van Heyningen, R. (1962). *Biochem. J.* **83**, 274.
- Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.
- Wilcox, H. G. & Fried, M. (1963). *Biochem. J.* **87**, 192.
- Wilhelmi, A. E., Fishman, J. B. & Russell, J. A. (1948). *J. biol. Chem.* **176**, 735.