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The Production of Amino Acids by Cell Fractions, particularly Rat-Liver Mitochondria

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(*Received* 24 *July* 1962)

The uptake of radioactive amino acids into rat-liver-cell organelles has been widely documented (e.g. Roodyn, Reiss & Work, 1961), but there is little published work on protein breakdown.

Amino acid liberation from cell organelles has been observed (Ansell & Richter, 1954*a*, *b*; Dingle, 1961; Bartley, Sobrinho-Simões, Notton & Montesi, 1958) and Korner & Tarver (1957) have investi-

gated the release of amino acid from labelled organelle protein; Penn (1961) has shown the breakdown of mitochondrial protein in the presence of CoA. The papers of Ansell & Richter (1954*a*), Gianetto & de Duve (1955), Finkenstaedt (1957) and Dingle (1961) describe acid cathepsins, the first in rat brain and the last three in rat liver, and the others deal with neutral proteinases, conditions, however, varying greatly.

This work describes the effect of various standard incubation conditions on the release of amino acids by rat-liver subcellular fractions, mainly mitochondria. It also included a comparative study of the production of amino acids by subcellular fractions in various tissues and species.

MATERIALS AND METHODS

Special chemicals. Ninhydrin (laboratory-reagent grade) was obtained from British Drug Houses Ltd. Other chemicals were commercial products of A.R. standard, where available.

Experimental animals. Male rats of the white Wistar strain, weighing 200–300 g., were used unless otherwise stated.

Sheep and ox tissues were obtained from the abattoir, being removed from the animal as soon as possible after death and stored in ice.

Homogenization medium. Sucrose (0·25 M) was used for all preparations. The sucrose solutions were adjusted to pH 7–7·4 with potassium hydroxide, cooled to 0–2° and used within 48 hr.

The pH of unneutralized sucrose solutions was frequently as low as 3·5 and when they were used for mitochondrial preparations the ratio of mitochondrial water content (mg.) to mitochondrial dry wt. (mg.) (*I*/*M* ratio) was often above 3, implying that the mitochondria were swollen (Werkheiser & Bartley, 1957). Normally *I*/*M* ratios of 2·4–3 were obtained with neutralized sucrose solutions.

Cell fractionation. Rats were killed by stunning and beheading and their livers were removed immediately and placed in ice. After being minced in a Fischer mincer, the chilled liver was homogenized in a stainless-steel Potter–Elvehjem homogenizer in 4 vol. of cold 0·25 M-sucrose solution, and the suspension centrifuged for 10 min. at 2000 rev./min. (600*g*) in rotor 822 of the International refrigerated centrifuge (PR 2). The supernatant was stored in ice. The nuclear fraction was prepared by resuspending the precipitate in 1½ vol. of the original minced liver in sucrose and centrifuging for 5 min. at 1000 rev./min. (150*g*). The operation was repeated twice, and the washed residue (cell debris) was discarded and the combined supernatants were spun for 10 min. at 2000 rev./min. (600*g*). The precipitate comprised the nuclear fraction and was stored until use at 0°. The supernatants from the two centrifugings at 2000 rev./min. were then combined and centrifuged at 4200 rev./min. (2700*g*) for 20 min. The supernatant was stored in ice whilst the precipitate was washed twice by resuspending in 1½ vol. of sucrose and centrifuging for 10 min. in the four-place high-speed head of the centrifuge at 3000 rev./min. (13 250*g*). The 'fluffy layer' obtained from these washings was stored separately, as was the precipitate comprising the mitochondrial fraction. The supernatants from the washings plus the supernatant from the 4200 rev./min. centrifuging were then spun for 30 min. in the eight-place, 50 ml. head of the M.S.E. high-speed 17′ refrigerated centrifuge at 16 000 rev./min. (30 000*g*). The precipitate was the microsomal fraction, the supernatant being the soluble fraction.

Routine preparations of mitochondria. (i) Rat and sheep liver and sheep kidney. These were prepared in 0·25 M-sucrose, as in the total cell fractionation except that the nuclear washes were omitted. This is essentially the method of Werkheiser & Bartley (1957), the difference being a higher centrifugal force (13 250*g* as opposed to 8500*g*) for the mitochondrial washing. This allows a better removal of the pink 'fluffy layer'.

(ii) Rat and sheep brain. These mitochondria were prepared in 0·25 M-sucrose by the method of Bellamy (1959).

(iii) Sheep spleen. These mitochondria were prepared by the same method as used for rat liver except that the initial mincing was done in a Latapie mincer.

(iv) Ox heart. Mitochondria were prepared in sucrose from ox heart by the method of Crane, Glenn & Green (1956).

(v) Pigeon breast muscle. These sarcosomes were prepared by the method of Chappell & Perry (1953).

Preparation of submitochondrial particles from rat-liver mitochondria. (*a*) Disrupted mitochondria were prepared by disrupting mitochondria prepared in sucrose in a French pressure cell (Aminco Scientific Instrument Co., Maryland, U.S.A.). The suspension was then centrifuged for 15 min. in head 296 of the International refrigerated centrifuge at 4200 rev./min. (26 000*g*), yielding 'supernatant' and 'precipitate' fractions.

(*b*) Mitochondria prepared in sucrose were also disrupted by freezing and thawing eight times.

Incubations. Unless otherwise stated, these were carried out in Warburg flasks containing 1 ml. of 0·1 M-potassium phosphate buffer, pH 7·4, at 40°. The total volume in the flask was normally 4 ml. The centre well contained either 0·2 ml. of 2 N-potassium hydroxide or phosphorus for aerobic or anaerobic conditions respectively. The gas phase was either air or nitrogen. Oxygen, when used, gave exactly the same results as air. Nitrogen or oxygen each containing 5% of carbon dioxide were also used, where stated.

Amino acid extraction. The reactions were stopped and the amino acids extracted by the addition of 2 N-perchloric acid (final concentration 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-potassium hydroxide at 0°. Trichloroacetic acid (10%, w/v) was also used in duplicate extractions; the results did not differ significantly from those obtained with perchloric acid.

Chemical estimations. Amino acids were estimated by a modified version of Rosen's (1957) ninhydrin method.

Samples containing 0·01–0·3 μmole of amino acid were made up to 1 ml. with water. Cyanide–acetate buffer, 0·5 ml. (0·2 mM-potassium cyanide in 3 M-sodium acetate buffer, pH 5·2–5·4), and 0·5 ml. of a 3% solution of ninhydrin in 2-methoxyethanol (methylCellosolve) were added and the samples placed in a boiling-water bath for 15 min. On removal the samples were made up rapidly to 7 ml. with 50% (v/v) ethanol. After cooling, extinctions of the samples were measured against a reagent blank at 570 mμ. Glutamate standards containing 0·1, 0·2 and 0·3 μmole were also measured. Hydroxyproline and proline, whose

ninhydrin reaction products have a maximum at 440 mμ, could not be detected when tested for qualitatively by the isatin method of Springall (1954). Ammonia, which gives a colour with ninhydrin, was estimated by microdiffusion (Conway, 1947). It comprised less than 1% of the total ninhydrin-positive material in 12 samples from five separate incubations.

Peptides, as shown by ion-exchange chromatography (Moore & Stein, 1951), were less than 1% of the total ninhydrin-positive material in three separate analyses, and were not detectable by using paper chromatography or high-voltage electrophoresis.

Tissue dry weights and water content. Mitochondrial water content was measured by pipetting a sample of mitochondria into a weighed Pyrex centrifuge tube and centrifuging at 0° in the high-speed head no. 295 of the International refrigerated centrifuge (PR2) for 10 min. at 4200 rev./min. (26 000*g*). The supernatant was decanted and the inside of the tube dried with strips of filter paper. After weighing, the mitochondria were treated with 10% trichloroacetic acid, washed twice with water and dried overnight at 105°.

Measurements of pH. All measurements of pH were made with the Pye Cambridge pH meter and a glass electrode.

RESULTS

Mitochondria have been the main particulate fraction used in this study. They were chosen for two reasons: because of the observations of Bartley *et al.* (1958) that washed particles of rat liver produced 0·02–0·03 μmole of glutamate/mg. dry wt./hr. on incubation anaerobically without substrate at pH 7·4, and because of their sensitivity to external conditions.

Endogenous amino acids and water content of rat-liver mitochondria. In 78 preparations the endogenous amino acid content of rat-liver mitochondria was between 0·011 and 0·085 μmole/mg. dry wt., mean 0·048 (54 of the values lying between 0·036 and 0·055). In 50 preparations the ratio of water content to dry weight (I/M) lay between 2·05 and 4·29, mean 2·92.

Production of amino acids in rat-liver mitochondria aerobically and anaerobically in the absence of added substrate. With mitochondrial preparations incubated at 40° with potassium phosphate buffer (pH 7·4 and final concentration 25 mM) containing sucrose (final concentration 0·188 M) for 1 or 2 hr. the mean amino acid production was 0·152 μmole/mg. dry wt./hr. ($q_{\text{amino acid}}$) anaerobically (32 samples, range 0·070–0·300) and 0·144 aerobically (29 samples, range 0·061–0·221). The use of nitrogen or oxygen containing 5% of carbon dioxide as the gas phase had no significant effect.

The difference between the two means is probably not significant.

Influence of time and temperature on amino acid production by rat-liver mitochondria. Fig. 1 shows the effect of incubating mitochondria aerobically and anaerobically at different temperatures for varying lengths of time. Amino acid production is proportional to time. This also occurs over a 5 hr. period anaerobically at 40° and over an 80 hr. period at 0°.

Influence of preincubation at 0° *on amino acid production by rat-liver mitochondria at* 40°. Storage of mitochondria at 0° before incubation at 40° increases amino acid production. Fig. 2 shows that the increase is somewhat greater when the incubation at 40° is anaerobic. After 2 hr. storage at 0°

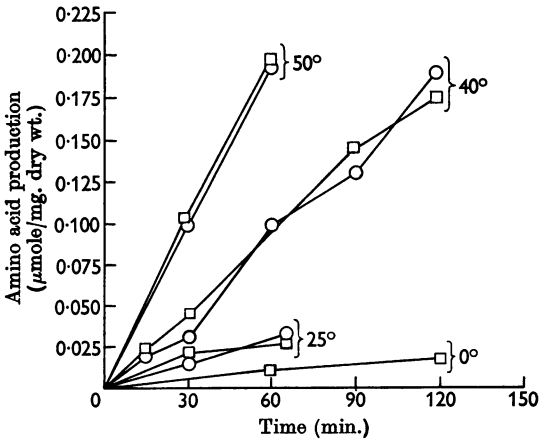

Fig. 1. Comparison of rates of amino acid production from rat-liver mitochondria at different temperatures. □, Aerobic incubations; ○, anaerobic incubations. The incubation mixture was as described for phosphate in Table 1.

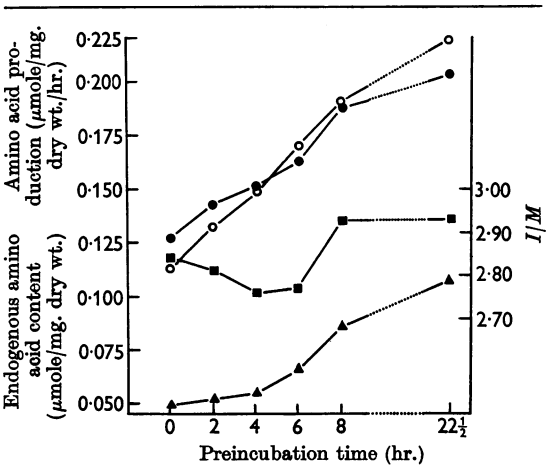

Fig. 2. Effect of preincubation of rat-liver mitochondria at 0° on subsequent amino acid production at 40°. ●, Amino acid production aerobically; ○, amino acid production anaerobically; ■, mitochondrial water content (mg./mg. dry wt.) before incubation at 40°; ▲, endogenous amino acid content before incubation at 40°. Incubations were for 1 hr. at 40° and the incubation mixtures were as described for phosphate in Table 1.

there was 13% increase in $q_{amino\ acid}$ on incubation aerobically at 40° and 17% increase anaerobically, and after 22½ hr. there was 73% increase aerobically and 131% increase anaerobically.

This pronounced effect of storage at 0° may explain in part the wide variation in $q_{amino\ acid}$, as mitochondrial preparation time becomes a critical factor.

Fig. 2 also shows the steady increase in amino acid content of the mitochondria at 0°. Thus the 'endogenous' amino acid content could indicate the extent of subsequent amino acid production, as it represents proteolytic activity during preparation of the mitochondria superimposed on the amount of the amino acid pool *in vivo*.

Effect of swelling of mitochondria on amino acid production. When mitochondria were swollen (*I*/*M* 3·2–4·5 before incubation at 40°) the $q_{amino\ acid}$ values were always high, giving a mean of 0·185 from eight anaerobic incubations and one of 0·170 from seven aerobic incubations.

Although swelling increases amino acid production, absence of swelling does not necessarily imply a low amino acid production (see Fig. 2).

Effect of breaking mitochondria, before incubation, on amino acid production. Table 1 shows that mitochondria, broken in the French pressure cell before incubation, have a considerably higher $q_{amino\ acid}$ than the control preparation. This is particularly noticeable in the presence of tris buffer (see below). Swollen mitochondria could thus be considered as partially broken mitochondria.

Table 1 also shows that the major part of the proteolytic activity is concentrated in the supernatant of the broken mitochondria, implying a soluble intramitochondrial enzyme or one that becomes soluble on disruption of the mitochondria.

Influence of tissue concentration in the incubation mixture on amino acid production. Amino acid production per unit dry wt. increases with increasing concentration of mitochondria, a fivefold increase in dry wt. per vessel causing a 225% increase in $q_{amino\ acid}$ (Table 2). This may explain in part the difference in $q_{amino\ acid}$ values in different experiments. It also implies some particle–particle interaction. Duplicate experiments have shown that the change in $q_{amino\ acid}$ with tissue concentration is smallest with between 30 and 60 mg. dry wt. of mitochondria/vessel (8–15 mg. dry wt./ml. of incubation medium).

Influence of sucrose concentration in the incubation medium on amino acid production. Amino acid production aerobically and anaerobically by mitochondria prepared in 0·25 M-sucrose in the presence of varying concentrations of sucrose (final concentration 0·063–1·063 M) is shown in Table 3. Marked inhibition is seen with increasing sucrose concentration. The greatest changes in production were found with the lowest sucrose concentrations, little further inhibition being found above a sucrose concentration of 0·563 M.

This could be explained as an osmotic effect, since with the low sucrose concentrations the mitochondria will tend to swell and disintegrate, and the structure will be maintained better with the high sucrose concentrations. In agreement with this, Table 4 shows that increasing sucrose concentration causes only a slight inhibition of the amino acid production by disrupted mitochondria.

Production of amino acids by rat-liver mitochondria aerobically and anaerobically in the presence of substrate. (*a*) Aerobic incubation at 40° for 1 hr. Several substrates have been used aerobically (final concentration 10–12·5 mM). Amino acid production is lowered with all but glutamate. Table 5 shows the percentage decrease with each substrate.

(*b*) Anaerobic incubation at 40° for 1 hr. Anaerobically α-oxoglutarate (final concentration 12·5 mM) and succinate (final concentration 10 or 12·5 mM) have been used. The results are shown in Table 5 and are very variable. Other experiments show a relationship between amino acid production and the concentration of added succinate per unit dry wt. of mitochondria. Thus with low succinate concentrations there is inhibition of amino acid production.

Table 1. *Amino acid production by whole and broken (and fractions of broken) mitochondria*

The preparations were incubated aerobically at pH 7·4 for 1 hr. at 40°. The incubation mixture contained 3 ml. of fraction used in 0·25 M-sucrose and 1 ml. of buffer, to give a final concentration of 25 mM. Figures for protein content are from Bartley, Getz, Notton & Renshaw (1962).

Fraction	$q_{amino\ acid}$ In phosphate buffer	$q_{amino\ acid}$ In tris buffer	Protein content (% dry wt.)
Whole mitochondria	0·185	0·254	—
Broken mitochondria	0·254	0·555	—
Supernatant	0·209	—	68·2
Precipitate fraction	0·016	—	56·0

Table 2. *Effect of tissue concentration on the production of amino acids by rat-liver mitochondria*

Incubations were for 2 hr. at 40° in nitrogen. The incubation mixture was as for phosphate in Table 1.

Dry wt. of mitochondria incubated (mg.)	Amino acid produced (μmoles)	$q_{amino\ acid}$
16·05	3·88	0·121
32·1	10·64	0·166
48·15	17·84	0·185
64·2	27·28	0·212
80·25	43·28	0·270

Relation between amino acid production and gas exchange. There is no relation between amino acid production and rate of endogenous respiration. Table 3 shows the respiration in the presence of varying amounts of sucrose and here respiration is inversely proportional to amino acid production. This is probably more a relation with the structural form of the mitochondria than a direct relationship between the two processes.

Q_{O_2} values have been expressed on the basis of oxygen uptake during the whole period of incubation (Q_{O_2} 'total') and during the first 15 min. of incubation (Q_{O_2} 'initial'). Q_{O_2} 'initial' gives some idea of respiration whilst endogenous substrates are still available. In nearly all cases Q_{O_2} 'initial' is higher than Q_{O_2} 'total', an exception being aerobic incubation of mitochondria in 1·063 M-sucrose.

In the presence of substrate there is a marked increase in Q_{O_2} coupled with a diminution in $q_{\text{amino acid}}$ values. This suggests that either amino acid oxidation is stimulated or that amino acid incorporation into the mitochondrial protein is enhanced.

Influence of pH *of incubation on the production of amino acids by rat-liver mitochondria.* Table 6 shows the effect of changing the pH of the incubation medium on the production of amino acids aerobically and anaerobically at 40°. The pH values at the

Table 3. *Effect of sucrose concentration on the production of amino acids by rat-liver mitochondria, aerobically and anaerobically*

Q_{O_2} 'initial' is based on oxygen uptake during the first 15 min. of incubation. Q_{CO_2} or Q_{O_2} 'total' expresses the mean gas change over the whole incubation period. Total vessel volume, 4 ml.: 3 ml. of mitochondrial suspension plus 1 ml. of 0·1 M-phosphate buffer, pH 7·4. Incubation conditions were as described in Table 1.

Final concn. of sucrose (M)	$q_{\text{amino acid}}$		Q_{O_2}		Q_{CO_2}
	Aerobic	Anaerobic	Initial	Total	Total
0·063	0·241	0·210	3·12	1·79	0·91
0·125	0·201	0·152	3·16	1·78	0·64
0·188	0·186	0·148	3·74	2·13	0·88
0·313	0·163	0·118	4·11	2·51	0·54
0·438	0·148	0·084	4·25	2·44	1·02
0·563	0·135	0·073	3·98	2·39	0·98
0·625	0·138	0·075	4·70	2·67	0·66
0·813	0·127	—	5·07	2·89	—
1·063	0·119	0·057	4·32	2·73	0·41

Table 4. *Effect of sucrose concentration on the aerobic production of amino acids by whole or broken mitochondria*

Q_{O_2} 'initial' is based on oxygen uptake during the first 15 min. of incubation. Incubation conditions were as described in Table 3.

Final concn. of sucrose (M)	Whole mitochondria			Broken mitochondria		
	$q_{\text{amino acid}}$	Q_{O_2}		$q_{\text{amino acid}}$	Q_{O_2}	
		Initial	Total		Initial	Total
0·063	0·112	1·01	0·58	0·140	0·96	0·56
0·188	0·104	1·67	1·18	0·135	0·22	0·22
0·438	0·073	2·16	1·22	0·127	0·47	0·34
0·688	0·070	1·73	1·59	0·118	—	—
1·063	0·065	1·27	1·56	0·114	0·22	0·72

Table 5. *Effect of substrate on the production of amino acids by rat-liver mitochondria*

Incubation mixture: 2·5 ml. of mitochondrial suspension in 0·25 M-sucrose plus 1 ml. of 0·1 M-potassium phosphate buffer, pH 7·4, plus 0·5 ml. of substrate in water or 0·5 ml. of water. Incubation conditions were as described in Table 1.

Substrate	Concn. (mM)	Change from control without substrate (%)			
		Aerobic	No. of expts.	Anaerobic	No. of expts.
Succinate	12·5	−23 (±4)	3	−38 to +88	11
α-Oxoglutarate	12·5	−37 (±15)	3	−52 to +78	4
Pyruvate	10	−32	1	—	—
β-Hydroxybutyrate	10	−23	1	—	—
Glutamate	10	0	1	—	—

beginning and end of incubation are given, showing that all the mitochondrial suspensions have a pH somewhere between 6 and 7 after incubation. This is some reflexion of the intrinsic buffering capacity of mitochondria.

Lowering the pH causes an increase in amino acid production, the effect being greater anaerobically than aerobically. After 2 hr. incubation anaerobically with acetate buffer, pH 4·1, approximately half of the mitochondrial protein was hydrolysed. The proteolysis at low pH values is probably due to lysosomal acid cathepsin, which has a pH optimum of 2–3 but still retains 25 % of its activity at pH 5 (Gianetto & de Duve, 1955). Amino acid production varies greatly, depending on the buffer used (see below), and a 50 % greater $q_{\text{amino acid}}$ was obtained in the presence of acetate buffer than with phosphate buffer, pH 5·2, so that some allowance must be made for this difference in those experiments where phosphate buffer was replaced by acetate buffer at low pH values.

Amino acid production at pH 7·4 *in the presence of various buffers without added substrate.* Penn (1961) stated that phosphate and pyrophosphate totally inhibit the breakdown of exogenous protein by mitochondria. Also Truman & Korner (1962) have shown that for amino acid incorporation in mitochondria the sodium to potassium ratio is critical. 65 mM-Potassium with 5 mM-sodium is necessary for maximal activity whereas 70 mM-sodium with no potassium is inhibitory.

Therefore several buffers at pH 7·4, both phosphate-free and sodium- or potassium-free, were tested. The ratio of potassium to sodium had no significant effect. Expts. 1 and 2 (Table 7) showed that $q_{\text{amino acid}}$ values were highest with the nitrogen-containing buffers (appropriate blanks were run for the ninhydrin reactions) and the bicarbonate buffer, whereas pyrophosphate almost completely inhibited production. There was no relationship between endogenous respiration and amino acid production.

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

Mitochondrial suspension in 0·25 M-sucrose (3 ml.) and 1 ml. of 0·1 M-buffer was incubated aerobically and anaerobically for 1 hr.

Buffer	pH in vessel: Before incubation	pH in vessel: After incubation	$q_{\text{amino acid}}$: Aerobic	$q_{\text{amino acid}}$: Anaerobic
Sodium pyrophosphate, pH 8·5	8·5	8·45	0·037	—
Potassium–sodium phosphate, pH 8·0	7·75	6·65	0·064	—
	—	6·35	—	0·080
Potassium phosphate, pH 7·4	7·30	6·60	0·080	—
	—	6·75	—	0·092
Potassium phosphate, pH 6·5	6·65	6·09	—	0·239
Potassium phosphate, pH 5·8	6·20	6·39	0·280	—
Sodium acetate, pH 5·2	5·32	5·49	—	0·662
	—	5·42	0·496	—
Sodium acetate, pH 4·1	4·58	4·35	—	1·561
	—	4·51	0·987	—

Table 7. *Production of amino acids by rat-liver mitochondria in the presence of various buffers, aerobically and anaerobically, and aerobically with added oxoglutarate* (12·5 mM)

Total vessel volume, 4 ml.: 2·5 ml. of mitochondrial suspension plus either 0·5 ml. of water or 0·5 ml. of α-oxoglutarate plus 1 ml. of buffer, pH 7·4. Q_{O_2} and Q_{CO_2} were calculated as described in Table 3. Incubation conditions were as described in Table 1.

Buffer and final concn.	Expt. 1 Aerobic without added substrate: $q_{\text{amino acid}}$	Expt. 1 Aerobic without added substrate: Q_{O_2} Initial	Expt. 1 Aerobic without added substrate: Q_{O_2} Total	Expt. 1 (*a*) Aerobic with 12·5 mM-oxoglutarate: $q_{\text{amino acid}}$	Expt. 1 (*a*) Aerobic with 12·5 mM-oxoglutarate: Q_{O_2} Initial	Expt. 1 (*a*) Aerobic with 12·5 mM-oxoglutarate: Q_{O_2} Total	Expt. 2 Anaerobic without added substrate: $q_{\text{amino acid}}$	Expt. 2 Anaerobic without added substrate: Q_{CO_2} Initial	Expt. 2 Anaerobic without added substrate: Q_{CO_2} Total
Glyoxaline–HCl (25 mM)	0·320	8·22	3·06	0·138	20·65	21·40	0·211	0·74	0·32
Tris–HCl (25 mM)	0·256	9·72	4·66	0·166	20·87	22·17	0·135	0·0	0·0
$NaHCO_3$–CO_2 (15 mM)	—	—	—	—	—	—	0·127	—	—
Triethanolamine–HCl (25 mM)	0·233	8·31	4·57	0·143	23·92	23·48	0·119	0·49	0·15
Potassium phosphate (25 mM)	0·172	1·42	1·21	0·104	24·10	13·59	0·079	1·00	0·46
Sodium ββ-dimethylglutarate (25 mM)	0·086	4·32	2·34	0·098	25·47	28·83	0·049	1·98	1·30
Sodium pyrophosphate (25 mM)	0·022	0·84	0·30	0·022	1·82	1·47	0·021	0·39	—

Amino acid production at pH 7·4 *in the presence of various buffers and of α-oxoglutarate.* In the presence of α-oxoglutarate (final concentration 12·5 mM) amino acid production was reduced with all the buffers used, apart from pyrophosphate and ββ-dimethylglutarate (14 % increase). However, these last two gave the lowest basic $q_{\text{amino acid}}$ values.

Effect of varying phosphate and tris concentrations (pH 7·4) *on amino acid production by whole and broken mitochondria.* Fig. 3 shows that production of amino acids aerobically by broken mitochondria was inhibited linearly by phosphate, whereas production in whole mitochondria was inhibited to a much greater extent and non-linearly. Similarly, with varying amounts of tris buffer (Fig. 4) much greater inhibition of production was found with whole than with broken mitochondria.

Anaerobically, equivalent curves were obtained.

The whole mitochondria were much less swollen with high phosphate concentrations in the medium, which could partially explain the big drop in amino acid production.

Influence of age, sex and diet of the rat on amino acid production by rat-liver mitochondria. Neither sex nor age of the rats (120–350 g.) had a significant effect on amino acid production.

Anaerobically the $q_{\text{amino acid}}$ of the mitochondria of a rat starved for 24 hr., compared with the $q_{\text{amino acid}}$ of a parallel preparation from a fed animal, shows little difference, but aerobically starvation causes a 20 % drop in $q_{\text{amino acid}}$.

Amino acid production by rat-liver-cell fractions. Table 8 shows the $q_{\text{amino acid}}$ values obtained from

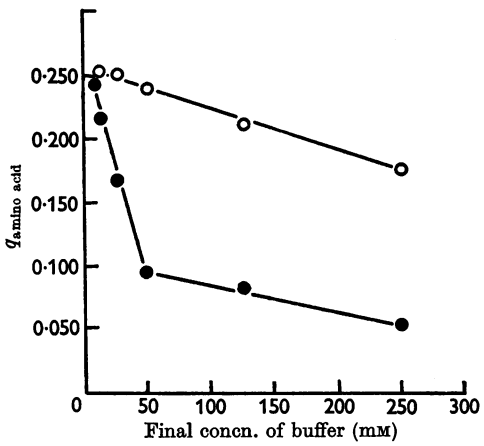

Fig. 3. Effect of the concentration of phosphate buffer in the incubation medium on the production of amino acids by whole and broken rat-liver mitochondria. Incubations were at 40°, aerobically for 1 hr. Incubation mixture: 3 ml. of mitochondrial suspension plus 1 ml. of phosphate buffer, pH 7·4, of appropriate concentration. ○, Broken mitochondria; ●, whole mitochondria.

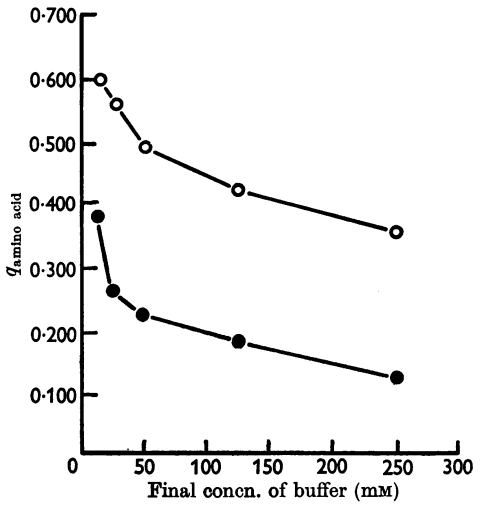

Fig. 4. Effect of the concentration of tris buffer in the incubation medium on the production of amino acids by whole and broken rat-liver mitochondria. Incubations were at 40°, aerobically for 1 hr. Incubation mixture: 3 ml. of mitochondrial suspension plus 1 ml. of tris buffer, pH 7·4, of appropriate concentration. ○, Broken mitochondria; ●, whole mitochondria.

Table 8. *Amino acid production by rat-liver-cell fractions at* pH 7·4 *and* pH 6·2

Incubations were for 1 hr.: 3 ml. of fraction in 0·25 M-sucrose was used plus 1 ml. of 0·1 M-potassium phosphate buffer, pH 7·4 or 6·2. Figures for protein content are from Getz & Bartley (1961).

Fraction	Protein recovery in each fraction (%)	$q_{\text{amino acid}}$ (pH 7·4) Aerobic	$q_{\text{amino acid}}$ (pH 7·4) Anaerobic	$q_{\text{amino acid}}$ (pH 6·2) Aerobic	$q_{\text{amino acid}}$ (pH 6·2) Anaerobic	Endogenous content (μmole/mg. dry wt.)	Protein content (% dry wt.)
Homogenate	100	0·103	0·103	0·054	0·039	0·132	77
Nuclei	21·6	0·373	0·244	0·072	0·039	0·070	84·2
Mitochondria	14·2	0·111	0·138	0·124	0·153	0·071	76·6
Microsomes	15·7	0·106	0·123	0·061	0·097	0·082	69·1
'Fluffy layer'	7·2	0·425	0·103	0·444	0·089	0·095	75·7
Soluble fraction	41·2	0·180	0·164	—	—	0·241	88·8

Table 9. *Production of amino acids, aerobically and anaerobically, with and without substrate, by mitochondria of different species and tissues*

Incubations were for 1 or 2 hr. at 40°. Mitochondria were made up to 3 ml. with 0·25 M-sucrose and incubated with 1 ml. of 0·1 M-potassium phosphate buffer. Succinate was added in 0·1 ml. of water. Numbers of experiments are shown in parentheses.

Species	Tissue	$q_{\text{amino acid}}$ Anaerobic	$q_{\text{amino acid}}$ Aerobic	$q_{\text{amino acid}}$ Aerobic + 10 mM-succinate	Endogenous content of amino acids (μmole/mg. dry wt.)
Rat	Liver	0·152 (32)	0·144 (29)	0·103 (5)	0·048 (78)
Rat	Brain	0·125	—	—	0·115
Sheep	Liver	0·093 (2)	0·019 (2)	0·015 (2)	0·051 (2)
Sheep	Brain	0·006	0·007	—	0·130
Sheep	Kidney	0·112	0·111	0·102	0·063
Sheep	Spleen	0·119	0·079	0·041	0·315
Ox	Heart	0·004	0·0	—	0·098
Pigeon	Breast muscle	0·035	0·012	0·015	0·027

various cell fractions at pH 6·2 and 7·4, together with the endogenous figures.

All fractions produced amino acids at both pH values. At pH 7·4 'fluffy layer' and nuclei had $q_{\text{amino acid}}$ 3·8 and 3·3 times that of mitochondria, whereas anaerobically nuclei had a 75% greater production than mitochondria. The microsomal fraction showed a very similar pattern to mitochondria. At pH 6·2 the $q_{\text{amino acid}}$ of the nuclear fraction was drastically reduced. 'Fluffy layer' still showed a high aerobic value but the mitochondrial fraction was the only fraction to show an increase in production. This value and the 'fluffy layer' value are probably due to activation of lysosomal cathepsin.

It is doubtful whether a single proteinase would cause these varying productions; two or more are implicated.

Amino acid production by mitochondria from other tissues and species. Mitochondria from various other tissues and species have been used. Table 9 shows the amino acid production by each of these. The results show that there is great variation between species, and also in different tissues of the same species.

Aerobically only rat liver and sheep kidney showed any notable amino acid production; anaerobically $q_{\text{amino acid}}$ values comparable with rat-liver mitochondria are obtained with all tissues used, apart from ox heart and sheep brain.

Endogenous values were highest in the two brain preparations. This, already noted for rat brain by Bellamy (1959), is due to a high endogenous glutamate concentration.

DISCUSSION

Amino acids have been found in varying amounts in the isolated mitochondria from many species and organs (see Bellamy, 1962). Our results are qualitatively in agreement with Bellamy's observation but our mean value for the initial amino acid content of the rat-liver mitochondria is some four times as high as his value. His results lie within the range of our values and, since we have shown that the production of amino acids by isolated mitochondria continues even at 0°, a range of values would be expected, depending on the length of time taken for the preparation of the mitochondria. Bellamy (1962) gives no values for the total amino acid of rat-brain mitochondria but if it is assumed that the proportion of glutamic acid + glutamine and aspartic acid of the total amino acids is the same in his analysis as in ours then the values are in essential agreement.

The mean amino acid production by rat-liver mitochondria at 40° was about 0·15 μmole/mg. dry wt./hr. The oxygen uptake of a similar mitochondrial preparation is about 0·2 μmole/mg. dry wt./hr., rising to about 2 μmoles/mg. dry wt./hr. when supplemented with succinate as substrate. Thus the production of amino acids is a process comparable in magnitude with the respiration in the rat-liver mitochondria, and in rat-brain mitochondria the rates of the processes become similar because of the higher amino acid production and lower rate of respiration. If the liberated amino acids were freely available for oxidation there would be sufficient substrate to maintain the endogenous respiration indefinitely. However, the liberated amino acids do not (with the exception of brain mitochondria; Bellamy, 1962) appear to be available for oxidation since the rate of production aerobically and anaerobically was about the same. Curiously, two of the main amino acids liberated from rat-liver mitochondria (glutamic acid and aspartic acid) are both oxidized by rat-liver mitochondria when added as substrate. The addition of glutamic acid to a mitochondrial preparation did not alter the liberation of amino acids from endogenous sources. It is not surprising that a wide scatter of absolute values was obtained because of the extreme sensitivity to conditions and the many

factors involved, such as sucrose and phosphate concentration, tissue concentration and pre-incubation time.

The liberation of amino acids from the intracellular structures may be due to the presence of active proteinases and peptidases or to a reversal of protein synthesis. The neutral proteinase activity of rat liver has not been extensively studied, although the liberation of amino acids from mitochondria has been noted by some workers (Korner & Tarver, 1957; Penn, 1961), and Waley & van Heyningen (1962) have compared the neutral proteinase activity in a number of tissues but after very different treatment. The cathepsin activity attributed to 'lysosomes' by Gianetto & de Duve (1955) has an optimum at pH 2–3 (Dingle, 1961), and should contribute little to breakdown at neutral pH. In addition its activity has only been tested on added protein. However, catheptic activity may contribute to increased protein breakdown at lower pH values but these need not be enzymes originating exclusively from the 'lysosomes'. Mitochondria and fluffy layer are the only two fractions which show bigger amino acid production at pH 6·2 than at pH 7·4 (Table 7), and these are the only fractions with any significant lysosomal content (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). In most isolated tissue fractions the amino acid production may well be due to the combined effects of several enzymes (neutral proteinases, peptidases and cathepsins), with contributions possibly from the reversal of the protein-synthetic pathways. Korner & Tarver (1957), measuring the liberation of labelled amino acids from their prelabelled mitochondria, found a sensitivity to the ratio of sodium and potassium in the medium that we have not observed, and Penn (1961) found that the proteolysis he was studying was inhibited by pyrophosphate, phosphate and bicarbonate, which we have found only with pyrophosphate. All these observations indicate more than one route of liberation of amino acids, any of which can predominate under different experimental conditions.

Whatever the enzymes concerned in amino acid liberation from intracellular organelles it is clear from the extent of the reaction that it must result in extensive modification of the structure of the organelle attacked. The liberation of amino acids is shown by all the fractions of a tissue homogenate but the activity of the reaction is very different in the different fractions, being maximal in the 'fluffy layer' and nuclear fractions (Table 7). Such differences in activity are not necessarily a reflexion of the intrinsic proteinase activity but may be conditioned by the presence of permeability barriers which partially segregate enzyme and substrate. This is illustrated in Table 1, where the activity of the mitochondria was doubled when the organelles were broken. In the intact tissues the rate of liberation of amino acids may be still further inhibited by segregation of enzyme and substrate. It may be calculated from the rate of production of amino acids in the liver homogenate that the whole of the tissue protein would be hydrolysed in about 59 hr. This value should be compared with the accepted half-life of the liver proteins (see, e.g., Schapira, Kruh, Dreyfus & Schapira, 1960) of 5 to 6 days. If a similar calculation is made for the 'intact' mitochondrial preparation it shows that these organelles would be completely hydrolysed in about 54 hr., whereas Fletcher & Sanadi (1961) have calculated a half-life of rat-liver mitochondria of about 10 days. It seems clear therefore that the neutral proteinases do not exhibit their full activity when in the intact cell. It can be seen that in the preparative procedure alone some activation (or removal from inhibition) takes place, because all rat-liver-cell fractions (Table 8) show higher proteolytic activity than the whole homogenate.

The influence of mitochondrial structure on the rate of production of amino acids is illustrated by the observation that a swollen preparation produced amino acids at a faster rate than a less swollen preparation. This disorientation of structure could allow easier contact of enzyme and substrate. The correlation between the content of free amino acids found in mitochondria and the rate of production is explained in the same way. It may be assumed that a high content of amino acids present in the mitochondrial preparation represents breakdown of mitochondrial substance during the preparation procedure. This has been shown to proceed even at 0° (Fig. 1), and the extent of this may well govern the ease of reaction between the enzymes and their substrates. The continued breakdown of mitochondrial structure by proteinases could explain the process of 'mitochondrial aging', since this phenomenon leads to increased permeability of mitochondria with loss of soluble small-molecular-weight components, uncoupling of phosphorylation and swelling.

The effects of substrates and buffers could also be explained in terms of changes in mitochondrial structure allowing less or more access of substrate to enzyme. However, the composition of the buffer also has a direct effect on the reaction; for example, in broken mitochondria amino acid production is twice as fast in tris buffer as in phosphate buffer. The increase in proteolytic activity in broken mitochondria also suggests that liberation of amino acids is faster when the proteinase has a bigger surface area of substrate to act on. The observations, showing that increased mitochondrial concentration increases the $q_{\text{amino acid}}$ (Table 2), infers that particle–particle interaction plays a considerable part in the reaction. In other words enzymes leaking out of one

mitochondrion will act on the outside of other mitochondria. If the absolute number of mitochondria within a given volume is increased, then the enzymes have a better chance of reaching suitable substrate, assuming that enzyme concentration is the limiting factor. Leakage of enzyme would also seem to be an important feature of preincubation at 0° (Fig. 2). This has an important effect on the subsequent $q_{\text{amino acid}}$ values obtained with aerobic or anaerobic incubation at 40°. Thus the longer the period of preincubation the larger the $q_{\text{amino acid}}$ obtained anaerobically compared with that obtained aerobically. This could explain the differences obtained between aerobic and anaerobic incubations in different experiments, as the preincubation is bound to vary in each particular case. The effects of preincubation also suggest a relation with the lysosomal group of enzymes (Appelmans, Wattiaux & de Duve, 1955), in particular the acid cathepsin. Preincubation causes an increase in the 'free' activity of these enzymes.

It is clear that the breakdown of the protein matrix of intracellular organelles when they are incubated *in vitro* is a factor that may complicate the interpretation of changes in amounts of amino acids when these are added as substrates, particularly when the period of incubation is prolonged. It is also a factor that must be taken into account when structural studies of mitochondria, for example, of swelling, are being made. The fact that amino acid production continues in rat-liver mitochondria at 0° implies that the initial state of such mitochondria will be variable and may explain the difficulty of comparing the results of different workers. The relatively low production of amino acids in heart-muscle mitochondria may explain the high stability of these mitochondria and their lack of swelling when compared with liver mitochondria.

Nearly all tissues investigated have shown some neutral proteinase activity and other workers have shown them in systems as diverse as calf lens (Waley & van Heyningen, 1962), embryonic cartilage (Lucy, Dingle & Fell, 1961) and clover leaves (Brady, 1961). Some of these enzymes are involved in special catabolic processes; thus Gordon & Humphrey (1961) have suggested that serum albumin is destroyed in rat-liver mitochondria *in vivo*, but the function of most of these enzymes would appear to be as degradative agents in the metabolic turnover of tissue proteins.

SUMMARY

1. Rat-liver mitochondria release amino acids (ninhydrin-positive material other than ammonia and peptides) when incubated at various temperatures and pH values. Anaerobically at 40° and pH 7·4, production was 0·152 μmole/mg. dry wt./hr. (range 0·070–0·300), and aerobically, 0·144 μmole/mg. dry wt./hr. (range 0·061–0·221). Amino acids were also liberated at 0° ($q_{\text{amino acid}}$ 0·012 aerobically). The production was linear with time.

2. The rate of amino acid production varied inversely with the solute concentration. The absolute rate depended on the nature of the solute, ranging from $q_{\text{amino acid}}$ 0·595 with 12·5 mM-tris to 0·055 with 250 mM-phosphate.

3. Amino acid production increased as the pH was lowered. It is suggested that amino acid production was due to different enzyme systems with different pH optima. Amino acid production varied with the type of buffer used in the incubation medium. The maximum rate was found with glyoxaline buffer ($q_{\text{amino acid}}$ 0·320 aerobically) and the minimum with pyrophosphate buffer (aerobic $q_{\text{amino acid}}$ 0·022).

4. Disrupted mitochondria gave higher $q_{\text{amino acid}}$ values than undamaged mitochondria (increase of 50–100%). This suggested that the proteolytic enzymes are soluble and intramitochondrial.

5. The presence of substrate aerobically lowered $q_{\text{amino acid}}$ values, the maximum inhibition of production being 50%.

6. All fractions of the rat-liver cell produced amino acids both aerobically and anaerobically at pH 7·4 and 6·2. 'Fluffy layer', incubated aerobically, was the most active fraction at both pH values.

7. Mitochondria from other tissues and species (rat brain; sheep liver, brain, kidney and spleen; ox heart; pigeon breast muscle) all produced amino acids when incubated under the same conditions as rat liver, to the greatest extent in rat brain (anaerobic $q_{\text{amino acid}}$ 0·125) and the least in ox heart (anaerobic $q_{\text{amino acid}}$ 0·004).

We wish to thank Professor Sir Hans Krebs, F.R.S., for his interest in the work. The work was aided by grants from the Rockefeller Foundation and the United States Department of Public Health. K.G.M.M.A. wishes to thank the Medical Research Council for a Training Scholarship.

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A Spectrophotometric Study of the Ionizations in Two Ferrihaemoproteins from Soya-Bean Nodules

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(*Received* 26 *February* 1962)

Ellfolk (1961) published pK values for the ionization of two ferrihaemoproteins isolated from soya-bean nodules grown in Finland. However, spectrophotometric data from comparable studies in America (Thorogood, 1955), noted only briefly in the literature (Thorogood, 1957) and since reinterpreted, indicate significant differences between the haemoproteins handled in the two Laboratories.

Several haemoproteins may occur in any soya-bean crop, but they can be cleanly isolated by paper electrophoresis. Commonly two major haemoproteins occur in about equal quantity. The present paper describes the visible- and ultraviolet-absorption spectra, in water, of the two major ferri-components, and also the unique changes in their visible- and near-ultraviolet-absorption spectra produced by alterations in the pH (from 4 to 10) of their buffered solutions, held at constant I (0·1). These spectra differ from those of Sternberg & Virtanen (1952), obtained on an unresolved mixture of the pigments, and from those of Ellfolk (1961), in that they reflect multiple haem-linked ionizations. The present paper considers the question whether so complex a titration pattern is suited to a pK analysis, and it details the experimental conditions that might render it so, and that have indeed been successful in subsequent thermodynamic analyses (Thorogood & Hanania, 1963).

A few months after the completion of this study, changes had occurred in the spectroscopic properties of the identical pigment preparations. The pigments failed to respond to changes of pH below pH 7. This response resembles that observed by Ellfolk (1961) and by Sternberg & Virtanen (1952) as the primary characteristic of their material, namely, a one-proton ionization. Analysis, however, reveals that the apparent monobasicity is an artifact, and raises the question whether the pigment of the Finnish workers is structurally different or whether theirs was aged, corresponding to the final product described in the present paper.

MATERIALS AND METHODS

Preparation of pigments. Temperatures were held to 15° or below during the preparation of the pigments. Block-tin-distilled water was used. Soya beans, *Glycine max*, var. Mammoth Yellow, were raised at the Morris Arboretum, University of Pennsylvania. Nodules (800 g.) were homogenized in 0·05M-phosphate buffer, pH 7, and the soluble extract was fractionated with ammonium sulphate. The pigment which was precipitated between salt concentrations of 26 and 32% (w/w) was dialysed free of salt and concentra-

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