The Localization of Proteolytic Activity in Rat Liver Mitochondria and its Relation to Mitochondrial Swelling and Aging

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1. On storage of rat liver mitochondria at 0°, water content, total amino acid content and leakage of protein all rose steadily over a 72 hr. period. The initial ratio of intramitochondrial to extramitochondrial amino acid concentration lay between 18 and 24. Initially this rose, but it then fell to 1.9 at the end of storage. The concentration gradient between internal and external amino acids was relatively constant throughout the period. These processes were accentuated at 22° and 40°, the concentration gradient reaching 70 μ moles/ml., water content rising to 8.3 mg./mg. dry wt. and protein leakage reaching 42% of total mitochondrial protein. 'Swelling agents' produced no correlated changes in amino acid production and swelling. 2. Added glutamate was not concentrated within the pellet of whole or disrupted mitochondria. Endogenous amino acids were distributed evenly between the pellet and the supernatant of disrupted mitochondria. It is concluded that amino acids are produced within mitochondria and that adsorption and uptake from the medium do not contribute significantly to amino acids in the pellet. 3. β -Glycerophosphate, a lysosome protectant, increased amino acid production by rat liver mitochondria. Treatment with Triton X-100 and disruption by freezing and thawing showed that 56% of proteolytic activity was 'free' in whole mitochondria, whereas only 11% of acid phosphatase activity, a lysosomal enzyme, was 'free'. 4. 'Light' mitochondria contained 30% more neutral proteolytic activity but 300% more acid phosphatase activity than 'heavy' mitochondria. 5. Electron micrographs of mitochondrial preparations showed less than one particle in 500 that could be identified as a lysosome. Treatment with Triton X-100 disrupted the structure of roughly 50% of the mitochondria; the rest appeared to retain their membrane, cristae and ground substance. Freezing and thawing caused gross swelling and loss of ground substance and rupture of external membranes. 6. Of the recovered proteolytic activity, 81% at pH 7.4 and 70% at pH 5.8 were found in the high-speed supernatant of broken mitochondria. A further fivefold increase in specific activity was found in the first protein fraction obtained by Sephadex G-50 gel filtration. 7. Between 60 and 80% of proteolytic activity was found in the 40–60%-saturated ammonium sulphate precipitate. Almost all of the soluble-fraction proteolytic activity could be recovered in a pH 5.0 supernatant. 8. The results give no support to the view that mitochondrial neutral proteolytic activity reflects lysosomal content. 9. The possible role of intramitochondrial amino acid production and the proteolysis of internal barriers in passive swelling of mitochondria is discussed.

It was shown by Alberti & Bartley (1963) that amino acids are produced by isolated rat liver mitochondria, even at 0°.

Lehninger (1962) divided mitochondrial swelling into two types: the first was a slow 'active' respiration-dependent type, and the second was a

rapid passive type in response to the osmolar concentration of relatively impermeable solutes in the medium. Tedeschi (1961) showed the latter to be reversible. In view of this and the observation by Avi-Dor (1960) that spontaneous swelling of rat liver mitochondria occurred on storage at 0°, it was decided to determine whether proteolysis resulted in an increased extramitochondrial amino acid

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concentration of sufficient magnitude to cause osmotic swelling.

Mitochondrial 'aging', the 'opening' phenomenon and their reversal were described by Green (1959). Aging has been attributed to the leakage of a mitochondrial protein, mitochrome (Polis & Shmukler, 1957), which causes uncoupling of oxidative phosphorylation and ATP breakdown. Internal proteolysis was considered to be the cause of leakage of this protein. The further documentation of protein leakage and its relation to amino acid production is recorded in this paper.

Proteolytic activity of rat liver mitochondria is increased by disrupting the mitochondria (Alberti & Bartley, 1965). This bears a similarity to the release of 'bound' activity from lysosomes (Gianetto & de Duve, 1955; Berthet & de Duve, 1951; reviewed by Weissmann, 1965). As lysosomes contain a large number of acid hydrolases, including cathepsins and a neutral arylamidase (Mahadevan & Tappel, 1967), investigation of the proteolytic activity of whole and disrupted mitochondria was necessary to determine the role, if any, of lysosomal enzymes in the production of amino acids by rat liver mitochondria at neutral pH. Baird (1964) was unable to rule out cathepsins as participants in amino acid production by broken mitochondria.

Integral in the discussion of both swelling and lysosomal participation is the actual localization of the neutral proteinases. This paper describes the relationship between mitochondrial swelling and amino acid production and also assesses the contribution of lysosomal cathepsin to mitochondrial hydrolysis. Fractionation of the mitochondria shows that most of the proteolytic activity is associated with the soluble portion of the mitochondria.

METHODS AND MATERIALS

Special chemicals. Ninhydrin (laboratory-reagent grade) was obtained from British Drug Houses Ltd., Poole, Dorset. 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 mitochondria and rat liver homogenate. These were prepared as described by Alberti & Bartley (1963). 'Heavy' and 'light' mitochondrial fractions were prepared by the method of Appelmans, Wattiaux & de Duve (1955).

Preparation of submitochondrial fractions. After disruption of normally prepared mitochondria in the French pressure cell, the resulting suspension was further fractionated by centrifuging at 13250g for 5 min. The supernatant was then centrifuged at 26000g for 15 min. Both these procedures were carried out in the four-place high-speed head of the International refrigerated centrifuge. The supernatant was then transferred to the eight-place 50 ml. head of the MSE High Speed 17 refrigerated centrifuge, and centrifuged at

16000 rev./min. for 1 hr. (1800000g-min.), and the supernatant was centrifuged again at the same speed for 2 hr. (3600000g-min.), the final supernatant being the 'soluble' fraction.

All precipitates were made up to an appropriate volume with 0.25M-sucrose and stored separately at -16° until used.

Gel filtration. Columns (2 cm. $\times$ 20 cm.) of Sephadex G-50 and G-25 were prepared by the method of Porath (1960). The retention volume of each column was about 18 ml., and fractions (1.5 ml.) were collected. The Sephadex G-50 columns were used in the fractionation of the 'soluble' fraction from disrupted mitochondria. Water was used as eluent. The Sephadex G-25 column was used to separate mitochondrial protein from $(\text{NH}_4)_2\text{SO}_4$ (Gelotte, 1960).

Ammonium sulphate fractionation. No precipitate was formed when mitochondrial soluble fraction was treated with $(\text{NH}_4)_2\text{SO}_4$ to 20% saturation at 0° . However, a large precipitate was formed at 40% saturation. This was centrifuged for 36000g-min., the supernatant was decanted, and the precipitate made up to 8 ml. with 0.025M-potassium phosphate buffer, pH 7.4. The $(\text{NH}_4)_2\text{SO}_4$ was then removed by gel filtration on Sephadex G-25. The supernatant from the 40%-saturation precipitation was taken to 60% saturation, giving a precipitate, and the supernatant from this was taken to 80% saturation, giving a further precipitate and leaving the final supernatant. The $(\text{NH}_4)_2\text{SO}_4$ was removed from the other precipitates in the same way as from the 40%-saturation precipitate. The fractions were stored at -16° before use. Ammonia was absent from all the fractions after gel filtration. The final supernatant contained only a small proportion of the total protein and so was not used in test incubations. Protein comprised 70–75% of the dry weight of all the fractions.

Precipitation at pH 5.0. The soluble fraction (3600000g-min. supernatant) of disrupted mitochondria was titrated to pH 5.0 at 0° with 0.1N-HCl, by using a pH-meter with an automatic temperature adjuster. After the pH 5.0 preparation had been allowed to stand for 1 hr. at 0° the precipitate and supernatant were separated by centrifugation. Both were then titrated back to pH 7.4 with 0.1N-KOH. An equivalent amount of KCl was added to the untreated soluble fraction as a control.

Preparative medium. 0.25M-Sucrose was used throughout.

Incubations. In 'swelling' experiments, mitochondria were incubated in 0.25M-sucrose with no added buffer. The pH of the preparation was between 6.8 and 6.9. Incubations of subfractions of mitochondria were carried out in conical flasks (10 ml.) in air, in a metabolic shaker. Other incubations were carried out at 40° in Warburg flasks. The centre well contained either 0.2 ml. of 2N-KOH, or phosphorus, for aerobic or anaerobic conditions respectively. The media are given in the Results section.

Amino acid extraction and mitochondrial water content. Normally the reactions were stopped and amino acids extracted by the addition of 2N-HClO₄ (Alberti & Bartley, 1963). When intramitochondrial amino acid concentration was measured, amino acid extraction and measurement of mitochondrial water content were performed simultaneously. Immediately after incubation, the mitochondrial suspension was centrifuged for 10 min. at 26000g in the four-place high-speed head of the International refrigerated centrifuge at 0° . The supernatant was decanted into 1 ml. of 2N-HClO₄. This was recentrifuged and the precipitate

discarded. This supernatant (E) contained the extra-mitochondrial amino acids. After careful wiping of the tube the original pellet was weighed and treated with exactly 1 ml. of 2*N*-HClO₄ and the pellet stirred with a fine glass rod. After weighing, the mixture was recentrifuged and the supernatant (I), containing the intramitochondrial amino acids, decanted. The pellet was dried and weighed and the water content of the original mitochondrial pellet calculated. The ratio of water content (mg.) to dry weight (mg.) (I/M) was used as a measure of swelling. This has been shown to be a more accurate measure of swelling than extinction changes (Bartley & Enser, 1964). The two supernatants, E and I, were neutralized with 2*N*-KOH and their amino acid contents measured. The ratio of internal (or pellet) amino acid concentration (P) to external (or supernatant) amino acid concentration (S) is referred to as the P/S ratio. No allowance has been made in the calculations for extramitochondrial water that remains interspersed in the original pellet after centrifugation. This amounts to about 20% of the mitochondrial-pellet water (Werkheiser & Bartley, 1957).

Chemical determinations. Total amino acids were measured by the method of Rosen (1957) as adapted by Alberti & Bartley (1963). Inorganic phosphate was measured by the method of Berenblum & Chain (1938) as modified by Bartley (1953). Ammonia was measured by direct Nesslerization (Harrison, 1939). The spectrophotometric method of Warburg & Christian (1942) was used for the determination of nucleic acid and protein. Protein concentration was calculated from the equation: $1.45 E_{280} - 0.74 E_{260}$ = protein concn. (mg./ml.). Nucleic acid content was read off a nomogram. Protein in (NH₄)₂SO₄ fractionations was measured by the biuret method (Tustanoff & Bartley, 1964).

Acid phosphatase activity. The method was adapted from that of Berthet & de Duve (1951). A sample (1 ml.) of tissue suspension was incubated with 1 ml. of 0.1*M* buffer containing 0.1*M*-sodium β -glycerophosphate at 40° for 10 min. at pH 5.0 or pH 7.4. The buffer comprised equimolar amounts of sodium acetate, borax and sodium cacodylate, adjusted to the appropriate pH with HCl ('ABC buffer' of de Duve, Berthet, Hers & Dupret, 1949). The reaction was stopped with 1 ml. of 10*N*-trichloroacetic acid. The precipitate was removed by centrifugation, and phosphate was determined in the supernatant. Rat liver homogenate and rat liver mitochondria were used either directly or after being frozen and thawed eight times to give 'free' and 'total' activity respectively. Activities at pH 5.0 showed good agreement with the values of Berthet & de Duve (1951). Measurements were also carried out at pH 7.4 to see whether comparable values would be obtained. All values were about 70% lower, and mitochondria contributed less to the total homogenate activity. Phosphatase measurements in subsequent experiments were therefore carried out at pH 5.0 as sensitivity was greater. Table 1 shows our values and those of Berthet & de Duve (1951).

Preparation of liver mitochondria for electron microscopy. Samples of mitochondria in 0.25*M*-sucrose at pH 7.0 were frozen and thawed three times or incubated with 0.1% of Triton X-100 at 0° for 50 min. Portions of these suspensions (0.5 ml.) and a sample of the original mitochondria were put in Spinco Microfuge tubes and centrifuged at 20000*g*_{max}. for 10 min. to produce firm pellets. After the supernatant had been removed, the pellets were fixed with 2.5%

Table 1. 'Free' and total acid phosphatase content of rat liver mitochondria and homogenate at pH 5.0 and pH 7.4

Suspension	pH of incubation	Free activity			Total activity		
		Phosphate released (μ g. of phosphorus/g. of original tissue/ 10 min.)	% of total homogenate activity	pH of incubation	Phosphate released (μ g. of phosphorus/g. of original tissue/ 10 min.)	% of total homogenate activity	Specific activity (μ g. of phosphorus/mg. of fraction — μ g. of phosphorus/mg. of homogenate)
Homogenate	5	339 (265)	20.4 (17.1)	5	1660 (1550)	100.0 (100)	1.0 (1)
	5	174 (87)	10.5 (5.6)	5	1231 (950)	74.2 (61.3)	4.9 (4.1)
Mitochondria	7.4	135	23.3	7.4	579	100.0	1.0
	7.4	61	10.6	7.4	292	50.3	3.4

The mitochondria and the homogenate were prepared in 0.25*M*-sucrose. Both suspensions were frozen and thawed eight times before incubation to give total activity, and were untreated before incubation for 'free' activity. For calculation of the acid phosphatase activity/g. wet wt. of liver, dry mitochondria were assumed to comprise of 5% the wet weight of liver (Werkheiser & Bartley, 1957). Values in parentheses are those obtained by Berthet & de Duve (1951) at 38° with 0.16*M*-ABC buffer. For details of the procedure see the Methods and Materials section.

glutaraldehyde in 0.09M-potassium phosphate buffer, pH 7.2, at 0° for 3 hr. The fixed pellets were removed from the Microfuge tubes and washed overnight in three changes of 0.25M-sucrose, pH 7.0, at 0-4°. The pellets were then post-fixed with 1% OsO₄ in 0.25M-sucrose for 2 hr. at 0°. After a washing in 0.25M-sucrose, pH 7.0, the pellets were dehydrated through a graded series of ethanol solutions followed by epoxypropene, and embedded in Araldite. Sections were cut with a Cambridge Huxley ultramicrotome, stained with lead citrate (Reynolds, 1963) and examined with an A.E.I. EM6B electron microscope.

RESULTS

Effect of storage of mitochondria at 0° and -16° on amino acid content, water content and protein leakage.

The total amino acid content and water content of mitochondria rose slightly over the first 4 hr. storage at 0°, and there was a small leakage of protein (Table 2). However, the distribution of amino acids between intramitochondrial and extramitochondrial compartments changed considerably. Initially the internal concentration rose by 25% and then fell steadily, the ratio of internal to external concentration (P/S) falling from 27.7 after 1 hr. to 5.9 after 8 hr. The same trends could be seen over a 72 hr. period, although no initial rise in P/S was seen, the first reading being at 12 hr. (Table 3). After 26 hr. the internal amino acid concentration rose. The concentration gradient was relatively constant.

Table 2. *Effect of storage of rat liver mitochondria at 0° on amino acid content, water content and protein leakage*

Mitochondria were stored at 0° in 0.25M-sucrose, and at the appropriate time a sample (3 ml.) was taken and centrifuged for 10 min. at 26000g. The supernatant was carefully decanted and both pellet and supernatant were treated with 1 ml. of 2N-HClO₄. After neutralization with 2N-KOH the amino acids in each fraction were determined. Protein leakage was measured gravimetrically. The dry weight at zero time was taken as 100%. I/M is the ratio of mitochondrial water content (mg.) to dry weight (mg.), and is used as an indication of mitochondrial swelling.

Incubation time (hr.)	Protein leakage (% of total)	I/M ratio	Total amino acid content (μmole/mg. dry wt.)	Amino acid concentration			P/S ratio	Concentration gradient (μmoles/ml.)
				In mitochondria (mm) (P)	Calculated on the basis of no swelling (mm) (S)	In medium (mm) (S)		
0	0.0	2.62	0.053	12.79	12.79	0.70	18.3	12.72
1	0.5	2.65	0.059	16.08	16.24	0.58	27.7	15.50
2	1.6	2.66	—	—	—	—	—	—
4	2.4	2.73	0.060	11.80	12.35	0.96	12.3	10.84
8	12.0	2.96	0.082	9.78	11.03	1.67	5.9	8.11

Table 3. *Effect of long-term storage of mitochondria at 0° and -16° on amino acid content, water content and protein leakage*

The conditions for the 0° samples were the same as those described in Table 2. The -16° samples were incubated individually in Pyrex tubes and thawed immediately before analysis. Determinations and annotations were the same as in Table 2. I/M is the ratio of mitochondrial water content (mg.) to dry weight (mg.), and is used as an indication of mitochondrial swelling.

Temp.	Incubation time (hr.)	Protein leakage (% of total)	I/M ratio	Total amino acid content (μmole/mg. dry wt.)	Amino acid concentration			P/S ratio	Concentration gradient (μmoles/ml.)
					In mitochondria (mm) (P)	Calculated on the basis of no swelling (mm) (S)	In medium (mm) (S)		
0°	0	0	3.07	0.056	11.9	11.9	0.5	23.8	11.4
	12	7.5	3.65	0.100	12.5	14.9	1.5	8.3	11.0
	26	14.1	3.76	0.178	10.4	12.6	3.7	2.8	6.7
	48	25.1	4.02	0.401	20.3	26.7	8.7	2.3	11.6
	60	29.8	4.88	0.582	25.0	39.7	12.8	1.9	12.2
	72	32.3	5.32	0.684	28.3	48.0	15.1	1.9	13.2
-16°	12	15.0	6.82	0.083	5.6	—	1.4	4.0	4.2
	26	23.8	6.44	0.104	8.1	—	1.7	4.7	6.4
	48	16.3	6.40	0.126	9.4	—	2.0	4.7	7.4
	60	21.4	7.42	0.102	6.5	—	1.8	3.6	4.7
	72	17.6	6.83	0.122	9.2	—	1.9	4.7	7.3

Table 4. *Effect of incubation for 1 hr. at 0°, 22° and 40° on amino acid content, water content and protein leakage of rat liver mitochondria*

Determinations were as described in Table 2. The 40° samples were incubated in a water bath and the 22° samples at laboratory temperature in air. Actual protein leakage at 0° was measured. Calculated concentration gradient is the internal amino acid concentration minus the external amino acid concentration, assuming that no swelling had occurred. I/M is the ratio of mitochondrial water content (mg.) to dry weight (mg.), and is used as an indication of mitochondrial swelling.

Temp.	Incubation time (min.)	Protein leakage (% of total)	I/M ratio	Total amino acid content (μmole/mg. dry wt.)	Amino acid concentration		P/S ratio	Concentration gradient (μmoles/ml.)	
					In mito- chondrial water (mm) (P)	In medium (mm) (S)		Actual	Calculated
0°	0	2.5	2.47	0.037	8.4	1.2	7.0	7.2	7.2
	15	4.6	2.45	0.041	7.5	1.3	5.8	6.2	6.1
	30	—	—	0.047	10.0	1.2	8.3	8.8	9.5
	60	9.4	2.48	0.057	10.0	1.8	5.6	8.2	8.2
22°	15	5.3	3.83	0.050	8.0	1.3	6.2	6.7	11.1
	30	16.5	3.33	0.061	9.9	2.1	4.7	7.8	11.2
	60	28.3	*	0.078	10.6	3.3	3.2	7.3	—
40°	15	34.4	5.67	0.108	25.5	1.7	15.0	23.8	56.8
	30	42.2	6.43	0.236	76.3	3.3	23.1	73.0	195.3
	60	34.0*	8.3*	0.526	81.5	12.3	6.6	69.2	259.9

* The pellet did not form well enough on centrifugation to allow accurate determination.

Protein leakage was as much as 32.3% of the total mitochondrial protein after 72 hr. incubation at 0°, and amino acid production accounted for a further 8–10% loss of mitochondrial protein. This implies a considerable derangement of structure.

Variable results were obtained at –16° (Table 3). After the first sampling there was no great change in amino acid content, P/S ratio, water content and protein leakage. Such once-frozen-and-thawed mitochondria are equivalent to mildly disrupted mitochondria. They have a high water content and can maintain only a low osmotic differential, and roughly one-fifth of their protein is lost to the medium.

Effect of incubation of mitochondria at 0°, 22° and 40° for 1 hr. on amino acid content, water content and protein leakage with and without swelling agents. At 40° there was a rapid increase in intramitochondrial amino acid concentration in the first 30 min. (Table 4). In the second 30 min. amino acid production was mainly reflected in an increase in the amino acid content of the extramitochondrial fluid. The P/S ratio fell from 23.1 to 6.6, although the concentration gradient was maintained at about 70 μmoles/ml.

Swelling was greatest after 1 hr. and became difficult to measure accurately. After 30 min. protein leakage already accounted for 42.2% of total mitochondrial protein. Despite the grossly swollen state of the mitochondria it is noteworthy that

amino acid accumulation within the mitochondria was faster than leakage out.

At 0° amino acid production was proportional to time and protein leakage continued steadily, reaching 9.4% after 1 hr. At 22° internal amino acid concentration rose steadily but proportionately less than the external concentration, so that the P/S ratio fell.

Only at 40° was there a large change in both water and internal amino acid content of mitochondria. Even at this temperature mitochondria maintained a large concentration gradient.

There was no direct correlation between amino acid production and swelling of mitochondria in the presence of calcium chloride (10 μM), thyroxine (10 μM), digitonin (1.0 μM), *p*-chloromercuribenzenesulphonate (10 μM) and 2,4-dinitrophenol (10 μM). Incubations were at 22° for 15 min. in the absence of buffer and it was concluded that active swelling was not related to amino acid production.

Distribution of endogenous amino acids and added glutamate in mitochondria suspended in 0.25 M-sucrose or water at 0° and 22°. Apart from intramitochondrial proteolysis the high P/S ratios noted above could be accounted for by either adsorption or active uptake of extramitochondrially produced amino acids. The distribution of added glutamate was therefore examined after 15 min. incubation of mitochondria at 0° (Table 5). Slight concentration of glutamate was found, but much less than would

Table 5. *Intra- and extra-particulate distribution of total amino acids in the presence and absence of glutamate in rat liver mitochondria suspended in 0.25 M-sucrose or water at 0° or 22°*

Determinations and analyses were carried out as described in Table 2. Included in the Table are the calculated distributions of glutamate allowing for the zero-time amino acid content and any amino acid production during the 15 min. of incubation, assuming that little change in location of endogenous amino acids occurred during this period. I/M is the ratio of mitochondrial water content (mg.) to dry weight (mg.) and was used as an indication of mitochondrial swelling. Total amino acid content was measured. It was assumed that significant metabolism of glutamate would not occur at 0° in 15 min. Thus total amino acid content with glutamate present includes 10 μ moles of glutamate.

Incubation medium	Temp.	Incubation time (min.)	Additions to medium	I/M ratio	Total amino acid content (μ moles)	Amino acid concentration		P/S ratio
						In mitochondrial water (mm) (P)	In medium (mm) (S)	
0.25 M-Sucrose	0°	0	None	2.83	3.27	12.10	0.75	16.2
	0°	15	10 μ moles of glutamate	2.69	14.27	21.74	5.66	3.8
	22°	60	None	3.03	6.53	11.62	2.55	4.6
			Glutamate sample minus zero-time:		10.79	8.75	5.12	1.7
			Allowing for extra 0.79 μ mole production:		10.00	6.20	4.79	1.3
Water	0°	0	None	9.62	4.29	4.23	1.70	2.5
	0°	15	10 μ moles of glutamate	8.87	16.79	11.28	7.34	1.5
	22°	60	None	8.51	20.25	13.55	9.61	1.4
			Glutamate sample minus zero-time:		12.50	8.20	5.72	1.4
			Allowing for extra 2.5 μ moles production:		10.00	5.35	4.75	1.1

Table 6. *Effect of β -glycerophosphate on amino acid production by rat liver mitochondria*

In Expt. 1 the incubation mixture consisted of 1 ml. of mitochondrial suspension in 0.25 M-sucrose made up to 3 ml. with sucrose, β -glycerophosphate or water to give the concentrations shown in the Table, plus 1 ml. of 0.1 M-potassium phosphate buffer, pH 7.4. Incubations were for 1 hr. at 40° in air. The control amino acid production was that of the mitochondria in 188 mm-sucrose (final concentration). In Expt. 2 the incubation mixture was 3 ml. of mitochondrial suspension in either 0.25 M-sucrose or 0.094 M- β -glycerophosphate, plus 1 ml. of 0.1 M-potassium phosphate buffer, pH 7.4. For this experiment mitochondria were prepared in 0.25 M-sucrose and then washed twice and made up to the required volume in either sucrose or glycerophosphate. Incubation conditions were as for Expt. 1 except that anaerobic incubations were carried out as well. 'Sucrose equivalents of β -glycerophosphate' represents osmotic equivalents; $\bar{\gamma}$ amino acid = μ moles of amino acid produced/mg. dry wt./hr.

Expt. 1

Final concn. of sucrose in incubation medium (mm)	Final concn. of β -glycerophosphate in incubation medium (mm)	Sucrose equivalent of β -glycerophosphate (mm)	Amino acid production (% of control)
188	0.0	0	100
181	2.4	7	107
156	12.0	32	110
156	0.0	0	118
125	24.0	63	114
63	47.0	125	122
63	94.0	250	124
63	12.0	32	139

Expt. 2

Washing and resuspension medium	Final concn. (mm)	$\bar{\gamma}$ amino acid		I/M ratio
		Aerobic	Anaerobic	
0.25 M-Sucrose	188	0.105	0.099	2.76
0.094 M- β -Glycerophosphate	71	0.111	0.098	4.04

allow the previous results to be explained by this mechanism. Adsorption was tested by examining the distribution of amino acids in mitochondria

disrupted by suspension in water. Distribution was again nearly equal between pellet and supernatant. It is therefore concluded that the amino acids are

Table 7. *Effect of Triton X-100 and freezing and thawing on amino acid production and acid phosphatase activity in the rat liver mitochondrial fraction*

Triton X-100 was used as a 0.1% solution in 0.25 M-sucrose. Incubations for amino acid production were for 1 hr. at 40°. The incubation mixture was 3 ml. of mitochondrial suspension plus 1 ml. of 0.1 M-potassium phosphate buffer, pH 7.4. Acid phosphatase activity was measured by the method described in the text. Total activity was taken as the highest activity obtained during each experiment. Broken mitochondria were prepared by freezing and thawing mitochondria eight times. Other details are as in Table 2. Protein concn. was 8–12 mg./ml.

Washing medium	Resuspension medium	Fraction and treatment after preparation	I/M ratio	Amino acid production (% of total)		Acid phosphatase activity (% of total)	
				Expt. 1	Expt. 2	Expt. 1	Expt. 2
0.25 M-Sucrose	0.25 M-Sucrose	Whole mitochondria	3.08	56	56	11	11
0.25 M-Sucrose	0.25 M-Sucrose	Broken mitochondria	—	100	100	81	100
0.25 M-Sucrose	0.1% Triton X-100	Whole mitochondria	4.16	99	57	48	—
0.25 M-Sucrose	0.1% Triton X-100	Broken mitochondria	—	68	91	100	—
0.1% Triton X-100	0.25 M-Sucrose	Whole mitochondria	4.79	30	—	32	—
0.1% Triton X-100	0.25 M-Sucrose	Broken mitochondria	—	49	68	38	27

produced within particles of the mitochondrial fraction.

Effect of β -glycerophosphate on amino acid production by rat liver mitochondria aerobically. β -Glycerophosphate has been used to prevent leakage of lysosomal enzymes (Appelmans & de Duve, 1955) and to protect nuclei from the hydrolytic action of lysosomal enzymes (Philpot & Stanier, 1956). Table 6 shows the effect of replacing sucrose in the incubation medium with progressively larger amounts of β -glycerophosphate, or of using β -glycerophosphate in the mitochondrial preparative procedure. If proteolysis was due to lysosomal enzymes then the β -glycerophosphate should decrease amino acid production. The opposite occurred: amino acid production increased with increase in β -glycerophosphate concentration in the incubation medium. The use of β -glycerophosphate in the preparation medium had little effect.

Effect of Triton X-100 and freezing and thawing on proteolysis and phosphatase activity in mitochondrial fractions of rat liver. Triton X-100 causes the release of bound hydrolytic enzymes from lysosomes (Wattiaux & de Duve, 1956). Freezing and thawing also causes lysosomal disruption as well as mitochondrial disintegration. Table 7 shows the effect of these two treatments on acid phosphatase activity, used as a lysosomal marker, and neutral proteolysis.

Freezing and thawing increased amino acid production twofold and acid phosphatase activity eightfold. The use of Triton X-100 in the resuspension medium, with or without freezing and thawing, caused a moderate increase in amino acid production. Freezing and thawing alone had a greater effect. Acid phosphatase activity was increased fourfold by the addition of Triton X-100 to the

resuspension medium, but ninefold when this was combined with freezing and thawing. The use of Triton X-100 in the washing medium caused a decrease in amino acid production in two out of three cases. It is possible that the freed proteinases were washed away in the final preparative procedure. Acid phosphatase activity was decreased by this treatment when compared with the effects of detergent in the resuspension medium with or without disruption, but was still greater than in the control undamaged mitochondrial fraction. The different behaviour of proteolytic and acid phosphatase activities suggests that they could be situated in two different types of particle within the mitochondrial fraction.

Amino acid production and acid phosphatase activity in rat liver homogenate and in 'heavy' and 'light' fractions of rat liver mitochondria after freezing and thawing. Amino acid production at pH 7.4 was 30% higher in the 'light' than in the 'heavy' fraction, whereas acid phosphatase activity was 210% higher in the 'light' fraction (Table 8). All activities referred to are total activities, as fractions were disrupted by freezing and thawing before incubation. Fig. 1 shows the results found here in the present work for acid phosphatase and neutral proteolytic activities compared with the findings of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) for acid phosphatase and acid cathepsin activities. The acid phosphatase results are in agreement, but de Duve *et al.* (1955) found much higher amounts of catheptic activity in the 'light' fraction compared with the neutral activity that we measured. This again suggests differences in distribution of acid phosphatase and neutral proteolytic activities. It is assumed that amino acid production is primarily the result of proteolysis,

Table 8. *Amino acid production and acid phosphatase activity in 'heavy' and 'light' mitochondrial fractions and in the homogenate of rat liver*

Both mitochondrial fractions and the homogenate were frozen and thawed eight times before incubation. Thus all activities are total. Incubation conditions were as described in Table 7. The values for percentage of total liver protein and percentage of wet weight of liver are based on the figures of Getz & Bartley (1961), who found 15% and 5% respectively for the total mitochondrial fraction. Values in parentheses are those of de Duve *et al.* (1955) based on the nitrogen distribution found here, and values in square brackets are the results obtained by de Duve *et al.* (1955), based on their nitrogen distribution, those in the proteolytic activity section being for cathepsin. Specific activity is equal to percentage of total activity divided by percentage of total liver protein. S.A.L. = specific activity of 'light' mitochondria; S.A.H. = specific activity of 'heavy' mitochondria; amino acid is defined in Table 6. Other details are as in Table 2.

Fraction	Proteolytic activity				Acid phosphatase activity							
	I/M ratio	% of total liver protein	% of liver wet wt.	Amino acid production (μmoles/g. wet wt. of liver/hr.)	% of total liver activity	Specific activity	S.A.L.	(μg. of P/ mg./10 min.)	(μg. of P/g. wet wt. of liver/10 min.)	% of total activity	Specific activity	S.A.H.
							S.A.H.					S.A.H.
Aerobic												
Heavy mitochondria	2.83	6.6 [16.3]	2.2 [5.4]	0.219	4.84 (14.0)	9.7 [35.0]	1.5 [2.8]	7.64	168.8	8.8 9.9 [24.1]	1.3 [1.5]	3.1 [3.7]
Light mitochondria	3.73	8.4 [7.4]	2.8 [2.5]	0.290	8.09 (48.0)	16.1 [43.0]	1.9 [5.8]	23.64	658.4	34.3 (45.4) [40.7]	4.1	—
Homogenate	—	100.0	33.3	0.149	50.00	100.0	1.0	6.92	1920.0	100.0	1.0	—
Anaerobic												
Heavy mitochondria	2.83	6.6	2.2	0.210	4.63	9.3	1.4	—	—	—	—	—
Light mitochondria	3.73	8.4	2.8	0.429	11.97	23.9	2.8	—	—	—	—	—
Homogenate	—	100.0	33.3	0.149	50.00	100.0	1.0	—	—	—	—	—

EXPLANATION OF PLATE I

Electron micrograph of mitochondria isolated by the procedure described in the text; magnification $\times 20000$. Most mitochondria have a well preserved matrix, outer membrane and cristae.

EXPLANATION OF PLATE 2

Electron micrograph of mitochondria isolated by the procedure described in the text and then exposed, at a mitochondrial protein concentration of 10mg./ml., to 0.1% Triton X-100 at 0° for 50min.; magnification $\times 20000$. Many of the mitochondria still retain matrix, outer membrane and cristae.

EXPLANATION OF PLATE 3

Electron micrograph of mitochondria isolated by the procedure described in the text and then frozen and thawed three times; magnification $\times 20000$. All the mitochondria show signs of change and most are grossly swollen.

Fig. 1. Acid phosphatase activity and proteolytic activity of the 'heavy' and 'light' fractions of rat liver mitochondria. The experimental conditions and results were those described in Table 8. ■, Acid phosphatase activity; □, proteolytic activity; 'de Duve', the results obtained by de Duve *et al.* (1955); 'Aerobic', results obtained in the present work under aerobic conditions; 'Anaerobic', proteolytic activity found in the present work under anaerobic conditions. The values for percentage of total nitrogen are taken from de Duve *et al.* (1955) as is the division of the total nitrogen into 'heavy'-mitochondrial nitrogen (16.3%) and 'light'-mitochondrial nitrogen (7.5%). Each column on the histogram shows two values for specific activity: A, activity of the 'heavy' mitochondrial fraction; B, activity of the 'light' mitochondrial fraction.

other mechanisms being insufficient to account for the magnitude of the reaction.

Electron-micrograph appearance of mitochondrial fractions of rat liver, freshly prepared, after detergent treatment and after freezing and thawing. Plate 1 is an electron micrograph showing the appearance of the mitochondria as isolated. The mitochondrial matrix is well preserved and hence the cristae are not so sharply defined as when the matrix is lost. Most of the mitochondria retain their double surrounding membrane. Examination of the field illustrated and 12 others randomly chosen showed only one particle that could be considered to be a lysosome, defined as being without cristae and showing the general appearance of the published pictures of lysosomes. The total number of particles examined was about 700. At a conservative estimate the contamination of the preparation with visible lysosomes was therefore less than 1%.

Plate 2 shows the state of the mitochondria after treatment with 0.1% Triton X-100 at 0° for 50 min. Many of the mitochondria are completely ruptured and empty of contents, but a substantial proportion retain their surrounding membrane and their contents. There is a rough parallel between the decrease in amino acid production that occurred

when the mitochondrial preparation contained Triton X-100 in the washing fluid and the decrease in the number of relatively undamaged mitochondria seen in the electron micrographs. Plate 3 shows the appearance of the mitochondria after being frozen and thawed three times. The mitochondria are grossly swollen; most have lost internal contents and structure and all have suffered some membrane damage. Again the appearance of the electron micrographs is in accordance with the stimulation of amino acid production that was found biochemically.

Amino acid production at pH 7.4 and pH 5.8 by subfractions of broken rat liver mitochondria, separated by differential centrifugation. In view of the increase in amino acid production observed on disruption of mitochondria, further subfractionation was carried out to localize the enzymic activity. Five fractions were prepared from broken rat liver mitochondria. The highest specific activity, defined as percentage of the total proteolytic activity/percentage of the total dry weight, at both pH 7.4 and pH 5.8 was found in the final (3600000g·min.) supernatant (Table 9). This fraction contained 51.1% of the total mitochondrial dry weight, 80.7% of the recovered proteolytic activity at

Table 9. *Amino acid production by subfractions of rat liver mitochondria, prepared by differential centrifugation*

Details of the fractionation procedure are given in the text. Precipitates from the centrifugation were made up to the required volume with 0.25M-sucrose. A sample (1.5 ml.) of each fraction was incubated with 0.5 ml. of 0.1M-potassium phosphate buffer, pH 7.4 or pH 5.8. Incubations were in air for 1 hr. at 40°. Reactions were stopped with 1 ml. of 2N-HClO₄.

Fraction	Dry weight (% of broken mitochondria)	Amino acid		% of total amino acid production		Specific activity (% of production) (% of dry weight)	
		pH 7.4	pH 5.8	pH 7.4	pH 5.8	pH 7.4	pH 5.8
Broken mitochondria	100.0	0.480	1.431	100.0	100.0	1.0	1.0
66250g-min. precipitate	6.2	0.108	0.60	1.4	2.7	0.2	0.4
390000g-min. precipitate	9.7	0.152	0.52	3.1	3.6	0.3	0.4
1800000g-min. precipitate	18.2	0.202	0.71	7.8	9.2	0.4	0.5
3600000g-min. precipitate	13.3	0.144	0.63	4.1	5.9	0.3	0.4
Final supernatant (soluble fraction)	51.1	0.64	1.39	68.6	50.3	1.3	1.0
Total	98.5			85.0	71.7

Fig. 2. Subfractionation of the soluble fraction of broken rat liver mitochondria, by using Sephadex G-50 column chromatography. A sample (6 ml.) of the soluble fraction was added to the column and eluted with water. Fractions A, B, C, D, E and F were collected from the effluent at the points marked. The fractions were subsequently tested for proteolytic activity (Table 10). Protein and nucleic acid contents of the effluent were calculated from the extinctions at 260 and 280 m μ . O, Protein content of effluent; ●, nucleic acid content of effluent. Quantities are given per ml. of effluent.

pH 7.4 and 70.2% of the recovered activity at pH 5.8. The specific activity of all other fractions lay between 0.2 and 0.5. The discrepancy between recovered and total proteolytic activity may have been due to partial separation of enzyme from substrate, non-specific soluble mitochondrial protein. Baird (1964) showed that some separation of enzyme and substrate was possible, although his conditions differed from ours.

In view of these findings the soluble fraction was used for further fractionations.

Amino acid production by subfractions of the mitochondrial soluble fraction obtained by gel filtration. The elution pattern of the mitochondrial soluble fraction on the Sephadex G-50 column is shown in Fig. 2. The parts of the effluent from which fractions A–F were taken are shown.

The soluble fraction used for the gel filtration

Table 10. *Amino acid production, at pH 7.4 and pH 5.8, by gel-filtration fractions of the soluble fraction of rat liver mitochondria*

Incubation conditions were as described in Table 9. The incubation mixture was 1.5 ml. of the fraction plus 0.5 ml. of 0.1 M-potassium phosphate buffer, pH 7.4 or pH 5.8. Definitions of the fractions and details of the fractionation procedure are given in Fig. 2. Protein and nucleic acid contents were calculated from the extinctions at 260 and 280 m μ . Fraction W was the soluble fraction of mitochondria before gel filtration.

Fraction	Dry wt. of incubated sample (mg./ml.)	% of total dry wt. in fraction	Amino acid production (μ moles/mg. dry wt./hr.)		Protein (mg.)/ nucleic acid (μ g.) ratio
			pH 7.4	pH 5.8	
A	6.2	9.3	0.79	1.37	4.4
B	2.8	8.7	0.150	—	5.8
C	3.1	12.1	0.017	0.32	2.9
D	1.8	19.2	0*	0.126	2.1
E	1.6	11.8	0*	0.000	1.5
F	1.0	6.5	0.130	0.310	1.3
W	6.4	100.0	0.164	0.75	—

* There was some diminution in ninhydrin-positive material in these vessels during incubation.

showed some diminution in amino acid production after storage for 3 weeks at -16° . Amino acid production was 0.64 μ mole/mg. dry wt./hr. at pH 7.4 and 1.39 μ moles/mg. dry wt./hr. at pH 5.8 before storage, and 0.164 and 0.75 μ mole/mg. dry wt./hr. at pH 7.4 and pH 5.8 respectively after storage.

Fraction A showed the highest proteolytic activity (Table 10). Activity at pH 7.4 was nearly five times that of the unfractionated soluble fraction after storage and 25% higher than before storage. Fractions B and C showed much lower activity. Fractions D and E both showed a decrease in ninhydrin-positive material during incubation at pH 7.4. Fraction F showed some activity at both pH values, but as this fraction contained only 6.5% of the total dry weight it contributes little to total activity. A greater proportion of neutral activity than of acid activity was found in fraction A, fraction C contributing nearly 30% of the acid activity.

Amino acid production at pH 7.4 and pH 6.4 by the soluble fraction of mitochondria after ammonium sulphate fractionation. At pH 7.4, phosphate buffer was 25 mM (final concentration), whereas it was 67 mM at pH 6.4. Thus the results at the two pH values are not strictly comparable, as it has been shown that buffer concentration influences amino acid production (Alberti & Bartley, 1963).

Two experiments were performed with ammonium sulphate fractions. Incubations were in air for 1 hr. at 40° . Specific activities varied but comparative changes were similar. Amino acid production was 0.41–0.46 μ mole/mg. dry wt./hr. in the 40–60% saturation precipitate compared with 0.25 for the whole soluble fraction of broken mitochondria at pH 7.4, and 0.34–0.41 compared

with 0.24 at pH 6.4. The 20–40% saturation precipitate showed no increase in specific activity over the control, and the 60–80% saturation precipitate showed no endogenous proteolysis at all.

Combinations of the precipitates were also incubated in case fractionation had separated enzyme from an endogenous substrate. All combinations showed slightly higher amino acid production than the predicted values.

Amino acid production at pH 7.4 and pH 5.8 by pH 5.0 subfractions of the soluble fraction of mitochondria. After pH 5.0 precipitation nearly all the recovered proteolytic activity at both pH values used for incubation was in the supernatant (100% at pH 7.4 and 85% at pH 5.8) (Table 11). Recombination of the subfractions at pH 7.4 gave a much lower yield of amino acids than the original soluble fraction had given.

DISCUSSION

The results obtained show that rat liver mitochondria are capable of sustaining a considerable concentration gradient of amino acids after incubation at 0° , 22° and 40° . There is considerable production of amino acids during this period. There are three possible explanations for the high intra-mitochondrial amino acid concentration: the first is that the amino acids are produced within the mitochondria, and that production exceeds leakage; the second is that adsorption of the extramitochondrially produced amino acids occurs; the third is that there is active uptake of amino acids from the medium. Adsorption would be expected to occur even with disrupted mitochondria: this was not found. Active uptake was not found when

Table 11. *Amino acid production by the soluble fraction of rat liver mitochondria after precipitation at pH 5.0*

Incubations were for 1 hr. at 40°. The incubation medium was 1.5 ml. of fraction plus 0.5 ml. of 0.1 M-potassium phosphate buffer, pH 7.4 or pH 5.8. Amino acid production is given in terms of μ moles of amino acid/mg. of protein/hr. The results are therefore 40% higher than when dry weight is used in the definition. P was the precipitate obtained after titration of the soluble fraction of broken mitochondria to pH 5 and S was the supernatant. Both were titrated back to pH 7.4 before incubation. W was the soluble fraction of mitochondria but with KCl added, equivalent to the amount used in the titration.

Fraction	Protein content (% of total)	Amino acid production (μ mole/mg. of protein/hr.)		Amino acid production (% of total)		Specific activity (% of total amino acid production % of total protein)	
		pH 7.4	pH 5.8	pH 7.4	pH 5.8	pH 7.4	pH 5.8
W	100.0	0.26	0.72	100.0	100.0	1.0	1.0
P	38.7	0*	0.270	0.0	14.2	0.0	0.4
S	61.3	0.31	0.90	74.5	80.0	1.2	1.3
		Total recovery		75.4	94.2		
P+S	100.0	0.080	—	31.1	—	0.3	—

* Diminution in ninhydrin-positive material during incubation.

glutamate was added to a mitochondrial suspension. Glutamate is known to be present in freshly prepared mitochondria (Bellamy, 1962; Baird, 1964) and increased amounts are found after incubation both aerobically and anaerobically at neutral pH (K. G. M. M. Alberti & W. Bartley, unpublished work; Baird, 1964; Bartley, Sobrinho-Simoes, Notton & Montesi, 1959). In addition, Garfinkel (1963) found that in low concentrations amino acids were able to enter rat liver mitochondria passively, but at higher concentrations penetration fell. Observations on rabbit heart mitochondria (Olson & Korff, 1967) showed that certain amino acids can be retained within the organelles for several hours at 0°.

It is possible that spontaneous swelling is related to the intramitochondrial accumulation of amino acids. This was suggested but not proved for pigeon breast muscle mitochondria by Harman & Kitiyakara (1955). Ox heart mitochondria and pigeon breast muscle mitochondria both have very low rates of amino acid production (Alberti & Bartley, 1963); Avi-Dor (1960) showed that ox heart mitochondria have a very low rate of spontaneous swelling, and Harman & Kitiyakara (1955) showed the same for pigeon breast muscle mitochondria. Sheep brain mitochondria also have a very low rate of amino acid production, but rat brain mitochondria show somewhat more active neutral proteolysis (Alberti & Bartley, 1963).

Werkheiser & Bartley (1957) showed that only 60% of intramitochondrial water was permeable to sucrose, giving an overall internal sucrose concentration of 150 m-molal. The sum of the concentrations of K⁺, Na⁺, Cl⁻ and phosphate was 120 m-molal, making the total internal solute concentration 270 m-molal. If swelling is regarded as an osmotic phenomenon, then at 0° with freshly

prepared mitochondria the amino acid concentration represents only an extra 4% osmolality. Even at 0°, amino acid production occurs and over a 72 hr. period the amino acid concentration gradient becomes constant despite a 70% increase in mitochondrial volume (Table 3), suggesting an equilibrium state. The calculated concentration gradient was 33 m-molar after 72 hr., representing a 12% increase in osmolality, or 15% if extramitochondrial particulate water is taken into account. Of swelling at 0°, 21% could therefore be accounted for by the osmotic effect of intramitochondrial amino acids.

The sucrose-impermeable space becomes sucrose-permeable during incubation or 'aging' (Bartley, 1961; Lehninger, Ray & Schneider, 1959; Tarr & Gamble, 1966). This could be a result of proteolysis of internal barriers and would account for a further 57% of swelling. Similar calculations for incubation of mitochondria at 40° for 30 min. show that 57% of swelling could be accounted for by the osmotic effect of intramitochondrial amino acids and a further 25% by breakdown of the sucrose barrier. It is not known whether amino acids are restricted to the osmotically active space. Values of 20–55% of the mitochondrial interior have been cited for this (Amoore & Bartley, 1958; Bartley, 1961; Tedeschi & Harris, 1955; Tarr & Gamble, 1966). The distribution of amino acids through the permeable and impermeable spaces would obviously alter their osmotic contribution. A morphological model for swelling has been proposed in which rupture of the outer membrane occurs with flattening of the cristae (Wlodawer, Parsons, Williams & Wojtczak, 1966; Blondin & Green, 1967). Amino acids would have to be concentrated within the inner membrane to maintain their concentration gradient and to participate in osmotic swelling if this model holds.

In the cases cited above about 80% of swelling

could be accounted for by internal amino acid production with breakdown of internal permeability barriers. Thus proteolysis may well contribute significantly to spontaneous swelling in 'iso-osmotic' media. Also explicable would be the slow spontaneous reversal of shrinkage induced by hyperosmotic sucrose (Bouma & Gruber, 1966). On the other hand rapid swelling is clearly unrelated to amino acid production and reversal of swelling cannot be accounted for by this mechanism.

Rosenthal & Rapoport (1961) demonstrated protein leakage from rat liver mitochondria and suggested that it was a chemical process not entirely dependent on swelling. The present work shows that protein leakage and amino acid production show parallel properties, more so than protein leakage and swelling (Table 3). It is suggested that proteolysis corresponds to the 'chemical process' of Rosenthal & Rapoport (1961). Leakage of protein is also responsible for the 'opening' phenomenon and mitochondrial aging. Both of these may be due to intramitochondrial proteolysis and the breakdown of protein barriers.

The contribution of lysosomal proteinases to neutral proteolysis in the mitochondrial fraction still remains to be elucidated. Baird (1964) was unable to reach a definite conclusion. Sawant, Desai & Tappel (1964) described the breakdown of mitochondria by lysosomes at pH 7.0, but their total activity was less than that shown here and they used a concentration of lysosomes ten times greater than the physiological concentration. An increase in proteolysis was found in the presence of β -glycerophosphate; lysosomes are protected by β -glycerophosphate (Berthet, Berthet, Appelmans & de Duve, 1951) and thus lysosomal proteolytic activity should be decreased. Results with Triton X-100 were more suggestive of an effect on two populations of mitochondria, one type being more prone to swell than the other (Werkheiser & Bartley, 1957) or being damaged in preparation (Ziegler, Lester & Green, 1956). Thus the latter would be particularly sensitive to detergents and the rest would respond to freezing and thawing as a normal population. The evidence presented in Plate 2 is consistent with this concept.

Similar proteolytic activity was found in the 'heavy' and 'light' mitochondrial fractions. On the other hand, we found a four times greater specific activity for acid phosphatase, a typical lysosomal enzyme, in the 'light' than in the 'heavy' mitochondria. The distribution of proteolytic activity was more consistent with typical mitochondrial enzymes (e.g. NADH-cytochrome *c* reductase; de Duve *et al.* 1955) than with lysosomal or micro-body enzymes. If acid phosphatase and neutral proteinases were concentrated in the same particle then the ratio of their specific activities

should have been the same in all fractions, whereas the ratio varied twofold. Proteolytic activity follows the pattern of several mitochondrial enzymes whose activity is doubled by freezing and thawing of mitochondria (Egger & Rapoport, 1963).

The intramitochondrial site of amino acid production, together with the failure of mitochondria to concentrate significant amounts of extramitochondrial amino acids, lends supportive evidence for the view that lysosomes are not responsible for the bulk of amino acid production at neutral pH. Assuming a value of one lysosome/50 mitochondria (Plate 1 suggests an even lower content) the concentration gradient would have to be 3.5 m-moles/ml. at 40°, sufficient to disrupt totally any organelle.

Part of the problem of differentiating between lysosomal and mitochondrial enzyme activities lies in the similarity of their behaviour to disruptive forces (Weismann, 1965). Sawant, Shibko, Kumta & Tappel (1964) showed that lysosomes respond to time, temperature and osmotic pressure with structural changes similar to mitochondria. The electron micrographs show that treatments commonly used for lysosomal rupture have some disruptive effect on mitochondria.

It is concluded that the results are more easily explained as effects on a broad population of mitochondria rather than on a small population of specialized particles. Lysosomal cathepsins must play only a small part in the neutral proteolytic reaction.

The major part of the proteolytic activity was concentrated in the soluble fraction of disrupted mitochondria, suggesting a soluble enzyme system or one that is loosely bound and freed by disruption. This is clearly differentiated from the protein-synthetic systems that are concentrated in the particulate fraction (Truman, 1963; Roodyn, Suttie & Work, 1962).

The 40–60%-saturated ammonium sulphate precipitate contained the most proteolytic activity. Aminopeptidase of ox lens (Wolff & Resnik, 1963) and ox spleen cathepsin C (Greenbaum & Fruton, 1957) were found in similar fractions. The results of gel filtration suggested a high molecular weight. Marks (1967) showed that neutral proteinases of brain are eluted first from Sephadex G-100 columns and followed later by acid proteinases.

Most of the mitochondrial neutral proteolytic activity was concentrated in the supernatant of a pH 5.0 precipitate. The results were similar to those obtained by Waley & van Heyningen (1962) with rat liver homogenate.

Therefore rat liver mitochondria contain a soluble neutral proteinase or proteinases, precipitable with 40–60%-saturated ammonium sulphate. They are separate from lysosomal cathepsins

and may be partly responsible for the spontaneous swelling of mitochondria.

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