The author is greatly indebted to Dr Knudsen, Courtauld Institute, Middlesex Hospital, London, W. 1, for carrying out the continuous electrophoresis and to Dr J. Bodman, 99 Harley Street, London, W. 1, for the starch-gel electrophoresis. Thanks are due to the Directors of The Crookes Laboratories Ltd. for permission to publish this work.

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Biochem. J. (1961) **82**, 218

The Endogenous Citric Acid-Cycle Intermediates and Amino Acids of Mitochondria

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(Received 3 August 1961)

It is well known that mitochondria isolated from a variety of animal tissues absorb oxygen when incubated without added substrate (e.g. Minnaert, 1960). Since a large proportion of the enzymes of the citric acid cycle are found in the mitochondrial fraction of the cell (Hogeboom, Claude & Hotchkiss, 1946; Schneider & Hogeboom, 1950; Chappell & Perry, 1953; Sacktor, 1955), it is probable that the endogenous substrates of mitochondria, if not citric acid-cycle intermediates, are capable of conversion into these compounds. Indeed, citrate has already been identified as an endogenous substrate of rat-liver mitochondria (Schneider, Striebig & Hogeboom, 1956). Other work (Minnaert, 1960; Weinbach, 1961) has shown that the endogenous respiration of rat-liver mitochondria is associated with the esterification of inorganic phosphate and may be inhibited by malonate and arsenite. Thus the available evidence supports the view that endogenous respiration involves substrate oxidation by way of the citric acid cycle.

This paper describes the extraction and measurement of citric acid-cycle intermediates and amino

acids in well-washed mitochondria obtained from a variety of animal tissues. It was found that all types of mitochondria contained glutamate and aspartate, whereas citric acid-cycle intermediates had a more limited distribution.

METHODS

Preparation of mitochondria. For details of the histological methods used to define the mitochondrial fraction of the cell see Bellamy (1958).

Rat and mouse liver. Mitochondria were isolated from rat and mouse liver by the method of Werkheiser & Bartley (1957), except that the dissolved CO₂ was not removed from the sucrose solution.

Rat and mouse skeletal muscle. The animals were killed by a blow on the head. The hind legs were skinned, severed at the pelvis and placed in ground ice. About 15 min. later muscle removed from the upper leg was passed through a chilled mincer. The mince was suspended in 10 vol. of 0.25 M-sucrose solution and homogenized (pestle moved up and down 10 times). The homogenate was centrifuged at 1000g for 10 min., the supernatant fluid decanted, stored in ice and the residue suspended in the original volume of 0.25 M-sucrose solution. The first centrifuging was then repeated. The combined supernatant fluids were centrifuged at 1000g for 10 min. The residue from this pro-

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cedure, consisting mainly of broken muscle fibres, was discarded. Mitochondria were sedimented from the supernatant fluid at 12 000g for 10 min. in a weighed tube.

Sedimented mitochondria were suspended in the isolation medium (equal to four times the volume of mince; 1 g. = 1 vol.) and centrifuged at 12 000g for 10 min. The procedure was carried out three times and the tube and sedimented mitochondria were quickly weighed. The yield of mitochondria was approximately 1% of the weight of mince.

Brain. Mitochondria were prepared from rat, mouse and pigeon brain as described by Bellamy (1959).

Pigeon breast muscle. Mitochondria were isolated from pigeon breast muscle by a method similar to that of Chappell & Perry (1953). The sedimented mitochondria were washed thrice by suspension in half the initial volume of 0.25M-sucrose solution and centrifuging twice at 3500g for 10 min. and finally at 25 000g for 10 min. The yield of mitochondria was approximately 5% of the weight of mince.

Frog skeletal muscle. The muscles were dissected from the hind legs of pithed frogs (*Rana temporaria*) and placed in ground ice. About 5 min. later the muscles were transferred to a chilled mortar, Ballotini beads no. 12 were added (half the original weight of muscle) and the tissue was gently ground with 0.25M-sucrose solution (vol. equal to 3 vol. of muscle) for 3 min. The mixture was then filtered through nylon cloth soaked in ice-cold 0.25M-sucrose solution. The residue was ground with fresh sucrose solution and the suspension filtered again. This process was repeated three times.

The combined filtrate was centrifuged at 650g for 10 min. to sediment the nuclei and muscle fibres. Mitochondria were isolated by centrifuging the supernatant fluid at 10 200g for 10 min.

The particles were washed three times in half the total volume of sucrose used for grinding the muscle; the suspension was centrifuged at 10 200g for 10 min. each time. The yield of mitochondria was about 2% of the whole muscle.

Mitochondria from invertebrate tissues

Locust. Mitochondria were prepared from the tissues of immature adult male and female locusts (*Schistocerca gregaria*) in 0.25M-sucrose solution as described by Bellamy (1958).

Crab and lobster hepatopancreas. The appendages of crabs (*Maea squinado*) and lobsters (*Homarus vulgaris*) were severed close to the body and the carapace was removed with bone forceps. The hepatopancreas was lifted out intact from the body cavity and placed in a beaker immersed in ground ice. After about 5 min. the whole tissue was homogenized (pestle moved up and down 10 times) with 5 vol. of 1.0M-sucrose solution. The fibrous cell debris was sedimented at 1000g for 10 min. and mitochondria were isolated from the supernatant fluid by centrifuging at 25 000g for 15 min. The sedimented mitochondria were washed thrice by suspension in half the original volume of 1.0M-sucrose solution and centrifuged at 25 000g for 20 min. The yield of mitochondria was about 6%.

Crab skeletal muscle. The appendages of shore crabs (*Carcinus maenas*) were severed close to the body and broken open with bone forceps. The muscle of the appendages was removed intact and passed through a chilled mincer (Spong Ltd.), weighed, suspended in 5 vol. of

1.0M-sucrose solution and homogenized (pestle moved up and down 10 times).

The homogenate was centrifuged at 1000g for 10 min. to sediment the muscle fibres and nuclei. Sucrose solution equivalent to the original volume used to suspend the mince was then added to the viscous supernatant fluid and the suspension centrifuged at 14 000g for 10 min. to sediment the mitochondria. Mitochondria were washed three times by suspending them in half the original volume of 1.0M-sucrose solution and centrifuging at 14 000g for 10 min. The yield of mitochondria was about 3% of the weight of the mince.

Earthworm body wall. Earthworms were immobilized by chilling in ground ice and the contents of the body cavity were removed. The body wall was washed in ice-cold sucrose solution (0.25M), chopped into small pieces and homogenized and centrifuged with the same procedure described for frog skeletal muscle. The yield of mitochondria was about 1% of the weight of the whole body wall.

Measurement of respiration

The Warburg technique was used with manometer flasks of total volume about 5 ml. Unless otherwise stated, the incubation medium contained 0.01M- K_2HPO_4 - KH_2PO_4 buffer, pH 7.4, and 2 mM- MgCl_2 (volume of incubation medium, 1.0 ml.). Substrate (10 mM) was added as indicated. Between 50 and 200 mg. wet wt. of mitochondria was added and the solute content adjusted to 0.25 osmolar (1.0 osmolar for mitochondria from marine invertebrates) with sucrose solution. The centre well contained 0.05 ml. of 2N-NaOH with filter paper. Incubations were at 25° with air as gas phase, and the uptake of O_2 was measured after a 5 min. equilibration period.

The initial rates of respiration are expressed as Q_{O_2} values ($\mu\text{l. of O}_2$ consumed/hr./mg. dry wt.). A comparison of the maximum Q_{O_2} values of mitochondria and homogenates is given in Table 1.

Dry weights were determined as 'acid-insoluble dry matter' by the method of Werkheiser & Bartley (1957).

Extraction and estimation of citric acid-cycle intermediates

Extraction. The technique described below was designed on the basis of experience obtained with several extraction procedures. The method is similar to that of Elliott (1954).

The mitochondrial suspension (containing between 100 and 500 mg. dry wt. in sucrose solution) was added to an equal volume of boiling ethanol in a 10 ml. plastic centrifuge tube, which was then loosely stoppered and placed in a water bath at 60°. Five minutes later 35% (w/v) NH_3 solution, equivalent to one-sixth of the volume of the suspension, was added and the mixture heated at 60° for a further 30 min. After the addition of the NH_3 solution the pH of the mixture was about 10 (measured with Universal indicator paper, British Drug Houses Ltd.). The mitochondrial suspension became translucent and particles of mitochondrial size (0.5–3 μ) could no longer be seen with the light-microscope. After incubation the pH was between 7 and 8, indicating that most of the free NH_3 had been evolved.

The mixture was centrifuged at 25 000g for 20 min. in a refrigerated centrifuge and the supernatant fluid, which contained the organic acids extracted from the mito-

Table 1. Comparison of maximum Q_{O_2} values of homogenates with those of mitochondria derived from them

Incubation conditions were as described in the text. All substrates tested (citrate, α -oxoglutarate, succinate, fumarate, malate and pyruvate) increased the endogenous O_2 uptake; the maximum Q_{O_2} values were given with α -oxoglutarate (Kg), succinate (S), pyruvate (Py) or malate (Ma). H, Homogenates; M, mitochondria.

Animal	Q_{O_2}								
	Glandular tissues			Nervous tissues			Muscular tissues*		
	H	M	M/H	H	M	M/H	H	M	M/H
Rat	9.0 Kg	23.0 S	2.6	7.3 Kg	18.6 Kg	2.6	14.7 Kg	0.6 S	0.4
Mouse	10.7 S	28.3 S	2.6	6.1 Kg	8.9 S	1.5	—	—	—
Pigeon	16.1 Py	6.0 S	0.4	7.9 Kg	17.2 Kg	2.2	12.4 Kg	15.0 S	1.2
Earthworm	—	—	—	—	—	—	0.7 S	4.5 S	6.4
Frog	—	—	—	—	—	—	0.9 Kg	2.8 S	3.1
Crab	2.3 S	6.3 S	2.7	—	—	—	4.3 Kg	14.8 S	3.4
Lobster	3.7 S	13.5 S	3.7	—	—	—	—	—	—
Locust	4.1 S	7.0 S	1.7	—	—	—	14.1 S	21.3 Ma	1.5

* Mitochondrial Q_{O_2} values stimulated up to threefold in the presence of 10 mM-ADP.

chondria, was poured into a 10 ml. tube. The dry weight of the residue was determined by the method of Werkheiser & Bartley (1957).

About 2 g. of moist Amberlite IRA-400 (passed through 30 mesh) in the carbonate form was added to the extract. The tube was stoppered and shaken for 10 min. in a Microid flask shaker (Griffin and George Ltd.) operated at maximum speed so that the resin was completely suspended in the extract. After shaking, the resin suspension was centrifuged at 2000g for 5 min. and the supernatant fluid discarded. The resin was suspended in water, transferred to a sintered-glass funnel and washed by successively stirring it in about 50 ml. of water until the suspending liquid was about pH 7.

The resin was mixed with 5 ml. of 10% HCl for 10 min. and then filtered. The resin was washed twice on the filter with 3 ml. of water and the combined filtrate freeze-dried. The residue, which contained the citric acid-cycle intermediates extracted from the mitochondria, was dissolved in 0.1 or 0.2 ml. of 50 mM- Na_2CO_3 solution and stored at 0° until the component acids could be estimated.

Estimation. Organic acids were separated by paper chromatography before estimation with the second solvent of Elliott (1954). The compounds were detected by lightly spraying the chromatogram with a solution of bromophenol blue (0.02% in 95% ethanol).

Each compound was eluted with 1 ml. of 50 mM- Na_2CO_3 solution at 60°. This solution was used for one of the following specific methods of estimation.

Succinate and fumarate were measured by a method of W. Bartley & B. Notton not published previously. The method involved the conversion of the two compounds into malate by the use of succinoxidase and fumarase, or fumarase alone. Malate was estimated by the fluorimetric method of Hummel (1949).

To measure succinate the procedure was as follows. The tissue sample, containing about 10 μ g. of acid, was adjusted to pH 6.8 with N-HCl. Sodium phosphate buffer, pH 6.8, was added to give a final concentration of 0.1M and the mixture was incubated for 1 hr. at 37° with 1 ml. of succinoxidase suspension [1 g. of succinoxidase (Krebs, 1937) in 10 ml. of 0.1M-sodium phosphate buffer, pH 6.8].

After 1 hr. the reaction mixture was cooled to room

temperature, 0.1 ml. of a solution of fumarase was added and the mixture maintained at room temperature for a further 10 min. The reaction was stopped by the addition of one-tenth of the volume of 50% trichloroacetic acid and the precipitated protein was removed by centrifuging. A sample (usually one-tenth of the total volume) of the supernatant fluid was taken for estimation of malate by the method of Hummel (1949). For the estimation of fumarate the sample was prepared in the same way except that the treatment with succinoxidase was omitted.

Straight-line calibration curves were constructed with standard solutions of malate; corrections were applied for the equilibrium of the fumarase reaction by addition of standard amounts of succinate or fumarate to the tissue extracts. About 85% of the fumarate was converted into malate. The fluorescence of the controls was 20–30% of that of the most concentrated standard solution of malate.

Citrate was estimated by the method of Saffran & Denstedt (1948).

α -Oxoglutarate was estimated by the method of Friedemann & Haugen (1943).

Extraction and estimation of amino acids

Amino acids were extracted and estimated as described by Krebs & Bellamy (1960). Glutamine was estimated with *Clostridium welchii* suspension (Krebs, 1948).

By using the above-mentioned methods, substrates added to rat-liver mitochondria at 0° immediately before extraction (similar in quantity to those present endogenously) were recovered within 90–96% (Table 2). When the methods were applied to other types of mitochondria the recovery of added substrates was not tested.

RESULTS

Endogenous substrates of mitochondria

Citric acid-cycle intermediates. Citric acid-cycle intermediates were detected in mitochondria from a variety of animal tissues (Table 3) but no common pattern of distribution was found. Mitochondria from pigeon brain, locust-head tissue and the

muscle of rat, pigeon, frog and earthworm apparently did not contain citric acid-cycle intermediates (detection limit about $0.2 \mu\text{mole/g. dry wt.}$).

Citrate. Citrate was the most abundant intermediate. It was found in mitochondria from lobster hepatopancreas, pigeon liver, rat liver and locust thoracic muscle (2.0 , 3.7 , 5.1 and $15.3 \mu\text{moles/g. dry wt.}$ respectively). The amount of citrate in rat-liver mitochondria was similar to that found by Schneider *et al.* (1956).

The endogenous citrate found in liver mitochondria by Schneider *et al.* (1956) was thought to arise because of the low activity of the enzymes immediately responsible for citrate oxidation. In the present work, although citrate produced the lowest stimulation of respiration in mitochondria from rat and pigeon liver (Q_{O_2} 3.2 and 4.5 respectively), this was not found with mitochondria from lobster hepatopancreas (Q_{O_2} 9.4) and locust thoracic muscle (Q_{O_2} 11.5 , associated with the highest concentration of endogenous citrate). Thus it may be said that the availability of substrate to the oxidative enzymes of mitochondria, and not the concentration of oxidative enzymes, is a major factor which influences the accumulation of endogenous substrates.

Other citric acid-cycle intermediates. Fumarate, α -oxoglutarate and succinate were also detected in mitochondria.

Fumarate was found only in mitochondria from rat and pigeon liver (1.4 and $0.5 \mu\text{moles/g.}$). Mitochondria from pigeon liver, rat brain and crab muscle contained α -oxoglutarate (1.2 , 0.4 and $0.4 \mu\text{moles/g.}$); crab-muscle mitochondria also contained succinate ($0.4 \mu\text{mole/g.}$).

Amino acids. All mitochondria contained glutamate and aspartate in amounts greater than those of the citric acid-cycle intermediates (Table 4). The highest concentrations of glutamate and aspartate were found in mitochondria from rat and pigeon brain, locust-head tissue and lobster hepatopancreas (range 10 – $70 \mu\text{moles/g.}$). Smaller quantities were found in mitochondria from rat and pigeon liver and the various types of muscle, with a concentration range 1 – $7 \mu\text{moles/g.}$ In mitochondria from liver and analogous tissues and muscle (except frog muscle), the ratio of glutamate to aspartate was less than 1.

Mitochondria from nervous tissue also contained glutamine. The total quantity of the three amino acids in brain mitochondria and the ratios of the amounts of the individual acids were similar for each type of mitochondrial preparation (the ratio of

Table 2. *Recovery of substrates added to suspensions of rat-liver mitochondria*

Various substrates (about $0.5 \mu\text{mole}$) were added to 3 ml. of a suspension of rat-liver mitochondria in 0.25M -sucrose and the suspension was extracted as described in the text. The suspension was found to contain the following endogenous substrates: citrate $0.53 \mu\text{mole}$, fumarate $0.19 \mu\text{mole}$, glutamate $0.52 \mu\text{mole}$ and aspartate $0.79 \mu\text{mole}$.

	Substrate (μmoles)			Percentage recovery
	Amount added	Amount found	Amount recovered	
Citrate	0.47	0.97	0.44	94
α -Oxoglutarate	0.51	0.49	0.49	96
Succinate	0.56	0.55	0.55	98
Fumarate	0.41	0.56	0.37	90
Glutamate	0.48	0.98	0.46	96
Aspartate	0.50	1.24	0.45	90

Table 3. *Endogenous citric acid-cycle intermediates in mitochondria*

The endogenous substrates were extracted from mitochondria (100 – 500 mg. dry wt.) with 50% ethanol containing 5% of NH_3 soln. and the extract was treated and chromatographed as described in the text. The detection limit for citric acid-cycle intermediates on a chromatogram was about $10 \mu\text{g.}$ of acid (about $0.2 \mu\text{mole/g. dry wt.}$). Figures refer to the experimental average.

		Substrate ($\mu\text{moles/g. dry wt.}$)					
		Liver and analogous tissues			Nervous tissues	Muscular tissues	
		Rat	Pigeon	Lobster	Rat	Locust	Crab
No. of expts. ...		3	2	1	5	4	1
Citrate		5.1	3.7	2.0	0	15.3	0
α -Oxoglutarate		0	1.2	0	0.4	0	0.4
Succinate		0	0	0	0	0	0.4
Fumarate		1.4	0.5	0	0	0	0
Malate		0	0	0	0	0	0

Table 4. *Endogenous amino acids in mitochondria*Endogenous substrates (μ moles/g. dry wt.) were extracted as described in the text.

	Substrate (μ moles/g. dry wt.)										
	Liver and analogous tissues			Nervous tissues			Muscular tissues				
	Rat	Pigeon	Lobster	Rat	Pigeon	Locust	Pigeon	Locust	Frog	Earth-worm	Crab
No. of expts. ...	5	5	3	6	3	2	4	4	1	1	1
Total free amino acids	12.4	8.6	—	—	—	—	2.5	1.9	—	—	8.7
Glutamate	4.3	3.8	25.4	75.2	46.9	30.8	0.7	0.7	4.6	1.5	2.9
Glutamine	—	—	—	37.5	25.7	19.2	—	—	—	—	—
Aspartate	6.8	4.0	43.6	27.3	10.5	9.3	1.1	0.9	1.3	2.3	5.7

Table 5. *Effect of pyruvate on the oxygen uptake of mitochondria*Incubation conditions were as described in the text. *E*, Endogenous Q_{O_2} ; *P*, Q_{O_2} in the presence of pyruvate. Figures refer to the mean $\pm$ S.E.M. of four experiments.

Animal	Q_{O_2}					
	Liver		Brain		Muscle	
	<i>E</i>	<i>P</i>	<i>E</i>	<i>P</i>	<i>E</i>	<i>P</i>
Rat	2.0 $\pm$ 0.1	8.3 $\pm$ 0.1	5.9 $\pm$ 0.5	11.4 $\pm$ 0.8	0.44 $\pm$ 0.15	0.50 $\pm$ 0.05
Mouse	0.8 $\pm$ 0.2	1.9 $\pm$ 0.3	2.9 $\pm$ 0.7	3.5 $\pm$ 0.2	0.53 $\pm$ 0.04	0.48 $\pm$ 0.01
Pigeon	2.5 $\pm$ 0.5	2.0 $\pm$ 0.1	3.6 $\pm$ 0.1	7.4 $\pm$ 0.3	0.6 $\pm$ 0.1	0.5 $\pm$ 0.2
Frog	—	—	—	—	0.8 $\pm$ 0.3	0.7 $\pm$ 0.4
Crab	3.4 $\pm$ 1.2	—	—	—	1.4 $\pm$ 0.3	1.6 $\pm$ 0.8
Lobster	6.3 $\pm$ 0.4	14.1 $\pm$ 1.9	—	—	—	—
Locust	1.4 $\pm$ 0.3	1.9 $\pm$ 0.3	0	—	5.6 $\pm$ 1.6	21.3 $\pm$ 3.8
Earthworm	—	—	—	—	0.7 $\pm$ 0.2	0.6 $\pm$ 0.3

glutamate to glutamine to aspartate was of the order of 3-4:2:1).

Glutamate and aspartate accounted for between 70 and 90 % of the total free α -amino nitrogen of some types of mitochondria. Paper chromatography of extracts of these mitochondria (Krebs & Bellamy, 1960) showed the presence of at least three other, less intense, ninhydrin-positive spots in addition to glutamate and aspartate.

Oxidation of endogenous amino acids in brain mitochondria

The endogenous respiration of mitochondria from rat brain and lobster hepatopancreas was unusually high (Table 5). The amino acid content of these mitochondria was also high (Table 4) and it is likely that the oxidation of endogenous free amino acids was responsible for the endogenous respiration. In this connexion it has already been demonstrated that the oxidation of free glutamate accounts for about 50 % of the endogenous oxygen uptake of brain slices (Takagaki, Hirano & Tsukada, 1957). The following experiments were carried out to investigate the endogenous metabolism of brain mitochondria.

Mitochondria from rat brain (56 mg. dry wt.) were incubated at 35° in the medium already

described, with no added substrate, until the uptake of oxygen could no longer be detected (1.5 hr.). Before incubation, the mitochondria contained 3.6 μ moles of glutamate, 1.8 μ moles of glutamine and 1.2 μ moles of aspartate. After incubation, no amino acids or citric acid-cycle intermediates were detected in the mitochondrial intersuspension. The oxygen uptake (34 μ moles) was only slightly higher than the theoretical value, which was calculated on the assumption that there was complete oxidation of the endogenous amino acids. Thus it appears that the oxidation of glutamate, glutamine and aspartate largely accounts for the endogenous respiration of brain mitochondria. The oxidation of glutamine probably requires its prior conversion into glutamate and suggests the presence of glutaminase in brain mitochondria. It is already known that glutaminase occurs in cell particles from rat liver (Blumsom, 1957).

When a similar experiment was carried out on liver mitochondria with a lower content of endogenous amino acids the situation was complicated by the production of glutamate and aspartate from an endogenous source. At the end of the incubation period the amount of glutamate and aspartate was about 70 % higher than that found initially. This finding is similar to that of Bartley, Sobrinho-

Table 6. *Effect of succinate on the endogenous amino acids of mouse-brain mitochondria*

Mouse-brain mitochondria (approx. 200 mg. wet wt.) were incubated for 60 min. as described in the text with and without succinate. The distribution of free amino acids between mitochondria and the incubation medium was determined by the method of Werkheiser & Bartley (1957). Before incubation the mitochondrial suspension contained 111 μ moles of α -amino N/g. (41 millimolal in the mitochondrial water).

	Amino acids	
	μ moles/g. dry wt.	Millimolal
No substrate		
Mitochondria	15.1	5.1
Medium	3.3	0.02
With 10 mM-succinate		
Mitochondria	74.7	21.9
Medium	22.6	1.1

Simoes, Notton & Montesi (1959) with liver homogenates and particle suspensions. It has since been confirmed with rat-liver mitochondria by K. G. M. M. Alberti & W. Bartley (unpublished).

The large increase in respiration after the addition of citric acid-cycle intermediates to brain mitochondria raises the question of a possible inhibition of the oxidation of endogenous amino acids by added substrate. In order to investigate this possibility the amino acid content of mouse-brain mitochondria was determined after incubation with and without succinate (which gave the greatest rate of oxygen uptake: Table 1). The results (Table 6) showed that the disappearance of endogenous α -amino nitrogen was considerably less in the presence of succinate.

DISCUSSION

The concentration of endogenous substrates in isolated mitochondria may be influenced by several factors, such as further oxidation of substrate during the isolation procedure, changes in substrate concentration if the equilibrium of a steady state is changed under the conditions of isolation, extraction of substrates by the washing procedure or adsorption of substrates from the soluble fraction of the cell. Any of these factors may have contributed to the present results.

The amounts of endogenous substrate in mitochondria were of the same order of magnitude as the amounts of potassium (40 μ moles/g. dry wt.), orthophosphate (12 μ moles/g.), organic phosphate (64 μ moles/g.) and pyridine nucleotides (3.7 μ moles/g.) that were found in rat-liver mitochondria by Werkheiser & Bartley (1957) and Birt & Bartley (1960). The concentration of the major inorganic cations of rat-liver mitochondria is about 150 milli-

molal, whereas the concentration of the balancing inorganic anions is about 100 millimolal (Amoore & Bartley, 1958). The large quantity of amino acids found in most types of mitochondria in the present work (equivalent to about 40 millimolal in the mitochondrial water) raises the possibility that these compounds account for about 10% of the internal osmotic pressure of the mitochondria. Indeed, the two- to three-fold increase in free amino acids which occurs on incubating rat-liver mitochondria without substrate suggests that the formation of free amino acids may be one factor which brings about mitochondrial swelling.

Several types of mitochondria were characterized by a high endogenous oxygen uptake, which was stimulated by pyruvate (Table 5). In other mitochondria pyruvate stimulated respiration only in the presence of exogenous malate. The former also had high concentrations of endogenous substrates. For example, rat-liver mitochondria contained moderately high concentrations of citrate, fumarate and amino acids; locust thoracic-muscle mitochondria contained large amounts of citrate and exceptionally large quantities of amino acids were found in mitochondria from lobster hepatopancreas and rat and pigeon brain. Endogenous glutamic acid was invariably found together with a similar quantity of aspartic acid. The two compounds are interconvertible, provided that the particle suspension contains transaminase associated with the enzymes of the citric acid cycle (Krebs & Bellamy, 1960). Therefore the predominance of glutamate and aspartate may be the result of a dynamic equilibrium involving the above-named enzymes. When brain mitochondria were incubated without added substrate endogenous glutamate and aspartate disappeared and no citric acid-cycle intermediates accumulated. These endogenous amino acids can apparently serve as an appreciable store of oxidizable substrate for mitochondria. The origin of the amino acids produced during the incubation of rat-liver mitochondria is not known, although the work of Bartley *et al.* (1959) suggests that they may be produced by the action of particle-bound cathepsins (see also de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955).

The mitochondrial fraction of some tissues contained no citric acid-cycle intermediates. This could be because enzyme concentrations are so adjusted that there is no accumulation of intermediates when mitochondria oxidize substrates. Other mitochondrial fractions contained predominantly citrate and fumarate. The presence of these intermediates only, particularly in view of the apparent oxidation of all exogenous substrates, indicates that they had limited access to the enzymes of the citric acid cycle.

In the previous discussion the mitochondrial fraction was tacitly assumed to be homogeneous, but the possibility must be considered that some citric acid-cycle intermediates are localized in particles which do not contain citric acid-cycle enzymes. It is now clear that the large-particle fraction of the cell contains particles with widely different properties [some, the lysosomes, with no apparent oxidative activity (Beaufay, Bendall, Baudhuin & de Duve, 1959)]. Particles of the lysosome type may have been included in the mitochondrial fractions isolated in the present work. The oxidation of endogenous amino acids in brain mitochondria, for example, may have occurred after the release of particle-bound amino acids which subsequently entered the mitochondria.

The presence of substrates in freshly isolated mitochondria, together with the possibility of the formation of substrate during incubation, must be considered when studies are made of the effects of exogenous substrate on mitochondrial respiration. In particular, the experiments with brain mitochondria, which suggest that exogenous succinate inhibits the oxidation of endogenous amino acids, may have a bearing on experiments with other types of mitochondria in which succinate was found to stimulate the reduction of endogenous mitochondrial pyridine nucleotides (Chance & Williams, 1955; Klingenberg, Slenczka & Ritt, 1959; Birt & Bartley, 1960). That is, the oxidation of exogenous succinate may involve an inhibition of the flow of electrons from endogenous substrates, possibly produced during the incubation, which are oxidized by way of DPN-linked enzymes.

SUMMARY

1. Washed mitochondria were extracted and analysed for citric acid-cycle intermediates. Endogenous glutamic acid and aspartic acid were also determined.

2. No endogenous citric acid-cycle intermediates were found in mitochondria from pigeon brain, locust-head tissue and the muscle of rat, pigeon, frog and earthworm (detection limit about $0.2 \mu\text{mole/g. dry wt.}$).

3. Citrate was found in mitochondria from lobster hepatopancreas, pigeon and rat liver and locust thoracic muscle (2, 4, 5 and $15 \mu\text{moles/g.}$ respectively). Fumarate, α -oxoglutarate and succinate were found in other types of mitochondria (range 0.4 – $1.4 \mu\text{moles/g.}$).

4. All mitochondria contained glutamate and aspartate (1 – $70 \mu\text{moles/g.}$). In some types of mito-

chondria glutamate and aspartate accounted for over 70 % of the free α -amino acid nitrogen. Mitochondria from nervous tissue also contained glutamine.

5. Experiments on the endogenous respiration of brain mitochondria indicated that amino acid oxidation was responsible for the endogenous oxygen uptake.

This work was carried out during the tenure of a Medical Research Council Scholarship for training in research methods and was aided by a grant from the Rockefeller Foundation. The author wishes to thank Professor Sir Hans Krebs, F.R.S., for his helpful criticism during the preparation of the manuscript and Dr W. Bartley for much discussion and advice during the experimental work.

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