The Permeability of Isolated Rat-liver Mitochondria at 0° to the Metabolites Pyruvate, Succinate, Citrate, Phosphate, Adenosine 5'-phosphate and Adenosine Triphosphate

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Studies on the distribution of solutes have shown that isolated rat-liver mitochondria are partially permeable to sucrose, sodium chloride and potassium chloride (Amoore & Bartley, 1958). In the work to be described, similar methods were employed to determine the permeability of mitochondria to certain anions of metabolic importance. Pyruvate, succinate, citrate, phosphate, adenosine 5'-phosphate and adenosine triphosphate were chosen, because there is presumptive evidence from the intracellular distribution of enzymes that in the course of metabolism these solutes probably traverse the mitochondrial membrane. For example, pyruvate formed by glycolysis in the soluble fraction of liver cells is destroyed by oxidation in the mitochondria (Siekevitz, 1957). Furthermore, there are indications from earlier work that the membrane may impose a permeability barrier upon the movements of these solutes. Thus Schneider, Striebich & Hogeboom (1956) showed that isolated mitochondria could retain their endogenous citrate if their structure was intact.

The distributions of solutes were measured at 0° to minimize the transformation of metabolites or active transport by the mitochondria. The distributions were determined at three external concentrations of the anion: mM, 10 mM and in the range 32–92 mM. The lowest concentration was intended to reveal any adsorption of the anion by the mitochondria; the middle concentration was taken as representative of those often employed in metabolic experiments *in vitro*; and the highest concentration was designed to measure the permeability of the mitochondria when any contribution by adsorption could be proportionately small.

METHODS

The general experimental procedures were as previously described (Werkheiser & Bartley, 1957; Amoore & Bartley, 1958), with certain modifications. Suspensions of rat-liver mitochondria were used as soon as the preparation was completed, so as to avoid the leakages of endogenous solutes that occur during storage of the stock suspension in 0·25M-sucrose at 0° (Amoore & Bartley, 1958). The sus-

pension of mitochondria in 0·25M-sucrose was mixed with different proportions of a 0·25 osmolar solution of the sodium salt of the anion whose distribution was to be studied. In Expt. 1 in each table (1–6) the suspension of mitochondria in 0·25M-sucrose was centrifuged without addition. In Expts. 2 and 3 sufficient salt solution was added to raise the concentration of anion in the medium to mM and 10 mM respectively. In Expt. 4 the concentration was made as high as possible (32–92 mM), consistent with maintaining the osmolarity. After 15 min. at 0° in contact with the salts, the mitochondria were centrifuged, and the mitochondrial pellet and supernatant fluid were analysed.

As some of the solutes are liable to transformation by the mitochondria, precautions were taken to ensure that the centrifuged mitochondrial pellets were kept at (or below) 0° until they had been thoroughly mixed with 10% (w/v) trichloroacetic acid. After centrifuging the mitochondria and decanting the supernatant fluid, the centrifuge tubes were drained, and the insides were dried with filter paper while the tubes were held in the cold air in the well of the International refrigerated centrifuge. The tubes were placed in an ice bath, 1 ml. of 10% (w/v) trichloroacetic acid was added, and the mitochondrial pellets were broken up with a glass rod. After about 1 hr. at 0°, the tubes were allowed to warm up to room temperature and were weighed, and the weight of the mitochondrial pellet was obtained by difference.

In experiments with adenosine triphosphate (ATP), the mitochondria were centrifuged through silicone fluid, with or without a lower layer of aqueous 'fixative' (Werkheiser & Bartley, 1957). When the mitochondria were suspended in mainly sucrose media a silicone fluid mixture of sp.gr. 1·055 was used, and with predominantly saline media a fluid of sp.gr. 1·035. The fixative contained about 0·5N-HClO₄ and 30% (w/v) of formaldehyde, and had a sp.gr. of 1·10. The mitochondrial pellets centrifuged without fixative were put, while still frozen, into 1 ml. of 10% trichloroacetic acid in a tared tube in an ice bath, and broken up as soon as they thawed.

Preparation of media

Succinic acid, citric acid, sodium citrate, NaH₂PO₄,2H₂O and Na₂HPO₄ (anhydrous) were obtained from British Drug Houses Ltd., and were of AnalaR quality. Sodium pyruvate was prepared as described by Bartley & Davies (1954). Adenosine 5'-phosphate (AMP) (monohydrate) was supplied by Zellstoff-Fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden, Germany. ATP (disodium adenosine triphosphate dihydrate) was obtained from Pabst

Laboratories, Milwaukee, Wisconsin, U.S.A.; paper chromatography showed ATP to be the only nucleotide present.

Glass-distilled water, freed from CO_2, was used for preparing the media. The phosphate medium (pH 7·4) was made by weighing out the appropriate amounts of mono- and di-basic salts and making up to volume. The succinate, AMP and ATP media were made by adding water to the solid salts or acid salt, and titrating with N-NaOH until they were dissolved and the pH was 7·4 (by glass electrode). The solutions were then made up to volume. The pyruvate and citrate media were made by dissolving the faintly alkaline sodium salts in the appropriate volume of water, and adjusting to pH 7·4 with small quantities of the corresponding acids. All media were calculated to be 0·25 osmolar.

Analytical methods

Sucrose. This was estimated by the method of Kulka (1956).

Sodium and potassium. These elements were estimated by means of a lithium internal-standard flame photometer (Amoore, Parsons & Werkheiser, 1958).

Phosphate. Orthophosphate was estimated by the method of Berenblum & Chain (1938) as modified by Bartley (1953). Total phosphate was estimated after wet-ashing the sample by the method of Hanes & Isherwood (1949), with the addition of 0·05 ml. of 5% (w/v) ammonium molybdate (Werkheiser & Bartley, 1957). The difference between total phosphate and orthophosphate was designated 'organic phosphate'.

Pyruvate. The 'specific extraction procedure' of Friedemann & Haugen (1943) was used, with halving of all volumes. It was found that the optical density decreased steadily after developing the colour with NaOH, and that the fading was accelerated by sucrose. Interference by the presence of up to 250 μmoles of sucrose/test was avoided by measuring the optical density within 3 min. of adding the NaOH to each sample. Pyruvic acid 2:4-dinitrophenyl-hydrazone was used to prepare a calibration curve.

Succinate. The method of W. Bartley (unpublished work) was employed. This involved conversion of succinate, by means of succinoxidase and fumarase, into malate, which was determined by the fluorimetric method of Hummel (1949). Any fumarate or malate in the original sample would be estimated as succinate.

Citrate. This was estimated by the method of Weil-Malherbe & Bone (1949), as modified by Taylor (1953). Up to 160 μmoles of sucrose/test did not affect recoveries of added citrate, but contributed a small blank.

Extraparticulate fluid. This was estimated by the method of Werkheiser & Bartley (1957) (see also Amoore & Bartley, 1958). [14C]Carboxypolyglucose (referred to below as 'labelled polyglucose') was added to the mitochondrial suspensions in a final concentration of 1% (w/v). The reproducibility of the estimations was substantially improved by decreasing the counting error, and by correcting for the self-absorption of the weak β-radiation (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). The counting error was reduced by accumulating a minimum of 7000 counts/sample, which represents a 'reliable error' of 2%. Self-absorption calibration curves were prepared by counting planchets containing equal amounts of labelled polyglucose, and increasing amounts of either sucrose or $NaClO_4$/Na_2CO_3, so that the surface density varied from

1·4 to 6·0 mg./cm.^{2}. (Drying for 5 hr. at 60° drove off all water and formaldehyde, and up to 95% of the trichloro-acetic acid.) The counting rate at a surface density of 3·6 mg./cm.^{2} was 10% lower with the salts than with sucrose. The counting rates of samples with different surface densities were corrected to the expected counting rate for an arbitrary surface density of 1·6 mg./cm.^{2}.

Calculations

The analytical results, calculated as previously described by Werkheiser & Bartley (1957), are given in the tables. The distribution of solutes between mitochondria and medium was expressed as the ratio of the internal and external concentrations. When this ratio was greater than unity, as was frequently observed for external salt concentrations of mM, it was taken to represent the 'degree of adsorption' of solutes by the mitochondria (Bartley & Amoore, 1958). With high salt concentrations the ratio was generally less than unity, and was expressed as a percentage, which was considered to represent the 'space' of the mitochondria accessible to an external solute (Amoore & Bartley, 1958). This 'space' is a measure of the permeability of the mitochondria to the given solute, and should be regarded as describing a steady-state distribution of solutes, rather than indicating the rate at which the movement of solutes occurred. The degrees of adsorption and the permeability spaces given below were calculated after subtracting the endogenous solute content of the mitochondria, indicated in Expt. 1, from the amounts found in Expts. 2–4. This subtraction was done so that the distribution of the added solutes could be obtained.

RESULTS

Distribution of sodium pyruvate between mitochondria and medium. The results are shown in Table 1. Freshly prepared mitochondria contained practically no endogenous pyruvate (Expt. 1), and the addition of mM-pyruvate to the medium caused no increase in the internal concentration (Expt. 2). Higher external concentrations of pyruvate caused the internal concentration to increase, but it remained below that in the medium. The distribution of sodium was rather similar, except that in Expt. 3 the concentration in the mitochondria rose substantially above that in the medium. There is no evidence for the adsorption of pyruvate, but some indication that sodium may be adsorbed. The spaces accessible to sodium and pyruvate were 75% and 61% respectively (Expt. 4), compared with a sucrose space of 60% (Expt. 1).

The considerable excess of the sodium space over the pyruvate space recalls the earlier finding of Amoore & Bartley (1958) that mitochondria exhibit a slightly greater affinity for sodium (or potassium) than for chloride. (With all the salts tested in this work, the sodium space was greater than the anion space.) The permeability to pyruvate appears to be about the same as that to sucrose. As with all experiments described in this

paper, the concentration of sucrose in the mito-chondria remained below that in the medium, although the concentration ratios varied. Low concentrations of sodium pyruvate had no effect on the potassium and total phosphate contents of the mitochondria, but in Expt. 4 it was noted that 9 % of their potassium and 6 % of their phosphate were lost. On a concentration basis the losses are apparently greater, owing to the increase in water content of the mitochondria from 1·54 to 1·80 l./kg. of mitochondrial dry weight (M).

Distribution of sodium succinate between mito-chondria and medium. The method of estimating 'succinate', as applied in this experiment, actually estimated the sum of succinate, fumarate and malate. It also gave a small reaction with AMP. The value of 5·8 millimolal (Table 2, Expt. 1) ob-tained for the endogenous 'succinate' concentration in the mitochondria was therefore treated as a blank without attempting to identify its components. Conversion of added succinate by the mitochondria into fumarate and malate would not be detected, but at 0° was probably negligible. When mm-sodium succinate was added to the medium (Expt. 2), large increases occurred in the sodium and especially the succinate concentrations in the mitochondria. The increments in concentration in the mitochondria showed degrees of adsorption of

2·2 for sodium and 10 for succinate. At an external concentration of 10 mM-succinate (Expt. 3) the concentrations of both ions were again higher in the mitochondria than in the medium, but in Expt. 4 the situation was reversed, indicating a sodium space of 53 % and a succinate space of 48 %, com-pared with a sucrose space of 60 % (Expt. 1).

The losses of potassium and phosphate from mitochondria exposed to the highest sodium succinate concentration (Expt. 4) were the same as those noted for the corresponding experiment with pyruvate (the same stock suspension of mito-chondria was used in Tables 1 and 2). The per-meability of the mitochondria to succinate was considerably less than that to sucrose or to pyruvate.

Distribution of sodium citrate between mitochondria and medium. The mitochondria contained 3·8 milli-molal endogenous citrate (Table 3, Expt. 1), which is in agreement with the finding of Schneider et al. (1956) that the citrate in rat-liver homogenates was mainly localized in the mitochondrial fraction. Addition of mM-sodium citrate caused considerable increases in both the sodium and citrate content of the mitochondria (Expt. 2). The degree of adsorp-tion was 2·1 for sodium and 8 for citrate. With 10 mM-sodium citrate in the medium (Expt. 3) the concentrations of these ions were still slightly

Table 1. *Distribution of sodium pyruvate between mitochondria and medium*

A concentrated suspension of mitochondria in 0·25m-sucrose (0·4 ml.) was added to 1·6 ml. of 0·25m-sucrose or 125 mM-sodium pyruvate, or mixtures of the two. Labelled polyglucose (0·2 ml. of 10 %, w/v, aqueous solution) was also added. After 15 min. at 0° the mitochondria were centrifuged at 25 000 g for 10 min. Analysis of the 125 mM-sodium pyruvate gave 120 mM-sodium and 117 mM-pyruvate. In this and other Tables, M represents mitochondrial dry wt.; the phos-phates are those extracted by 10 % (w/v) trichloroacetic acid; and the external concentrations of potassium (and of phos-phates except where stated) were less than 3 millimolal.

| | Internal water content of | Concn. of solutes (millimolal) | | | | | | | |
| | | Pyruvate | | Sodium | | Sucrose | | | |
Expt. no.	mitochondria (l./kg. of M)	External	Internal	External	Internal	External	Internal	Potassium. Internal	Total phosphate. Internal
1	1·54	<0·1	0·1	<0·1	1·4	233	139	75·1	74·8
2	1·47	0·8	<0·1	1·2	1·9	234	139	79·9	79·6
3	1·47	9·4	8·5	8·6	17·1	215	116	78·0	78·0
4	1·80	92·0	56·3	98·5	75·0	39·5	15·2	58·4	60·0

Table 2. *Distribution of sodium succinate between mitochondria and medium*

Details are as given in Table 1, except that 83 mM-sodium succinate was used instead of sodium pyruvate. The mito-chondrial suspension in 0·25m-sucrose was the same as that used in Table 1. Analysis of the 83 mM-sodium succinate gave 167 mM-sodium and 83·4 mM-succinate.

| | Internal water content of | Concn. of solutes (millimolal) | | | | | | | |
| | | Succinate | | Sodium | | Sucrose | | | |
Expt. no.	mitochondria (l./kg. of M)	External	Internal	External	Internal	External	Internal	Potassium. Internal	Total phosphate. Internal
1	1·54	0·5	5·8	<0·1	1·4	233	139	75·1	74·8
2	1·58	1·3	13·6	1·7	5·2	230	129	73·3	72·9
3	1·49	10·0	23·0	18·7	21·5	208	102	74·6	72·0
4	1·72	66·9	37·2	134	73·0	40·1	17·5	60·8	62·5

Table 3. *Distribution of sodium citrate between mitochondria and medium*

In Expts. 1–3, a suspension of mitochondria in 0·25M-sucrose (2 ml.) was added to the appropriate volume of 62 mM-sodium citrate. In Expt. 4, some of the original suspension in 0·25M-sucrose was centrifuged, and the mitochondria were resuspended in 62 mM-sodium citrate. A sample (2 ml.) of the resulting suspension was taken. Labelled polyglucose (0·2 ml. of 10%, w/v) was also added to each tube. After 15 min. at 0° the mitochondria were centrifuged. Analysis of the 62 mM-sodium citrate gave 184 mM-sodium and 61·1 mM-citrate.

Expt. no.	Internal water content of mitochondria (l./kg. of M)	Concn. of solutes (millimolal)							
		Citrate		Sodium		Sucrose		Potassium. Internal	Total phosphate. Internal
		External	Internal	External	Internal	External	Internal		
1	1·68	<0·1	3·8	<0·1	0·4	245	117	65·2	59·0
2	1·76	0·6	8·4	2·4	5·0	238	126	64·0	58·6
3	1·70	10·1	12·5	29·2	31·7	209	88·1	65·4	56·0
4	1·78	53·1	26·7	158	92·6	15·8	12·5	39·2	54·7

Table 4. *Distribution of sodium orthophosphate between mitochondrial pellets and medium*

Details are as given in Table 3, except that 89 mM-sodium phosphate was used instead of sodium citrate, and labelled polyglucose was absent. Analysis of the 89 mM-sodium phosphate gave 158 mM-sodium and 88·5 mM-orthophosphate.

Expt. no.	Total water content of pellet (l./kg. of M)	Concn. of solutes (millimolal)							
		Orthophosphate		Sodium		Sucrose		Potassium. Pellet	Organic phosphate. Pellet
		Supernatant	Pellet	Supernatant	Pellet	Supernatant	Pellet		
1	2·46	0·1	9·7	<0·1	<0·4	264	185	45·3	26·7
2	2·51	0·9	12·9	1·2	4·3	260	186	43·9	25·9
3	2·58	11·0	25·5	18·4	29·0	234	168	36·0	24·7
4	4·26	84·1	74·4	147	143	18·6	12·8	10·9	15·7

greater in the mitochondria than in the medium. In Expt. 4 the exclusion of sodium citrate from part of the mitochondrial volume was demonstrated, the concentrations of sodium and citrate in the mitochondria remaining substantially lower than those in the medium. The sodium space was 58% and the citrate space 43%, compared with the sucrose space of 48%. The permeability of the mitochondria to citrate was somewhat less than that to sucrose. The sodium citrate medium (Expt. 4) had no effect on the phosphate content of the mitochondria, but 36% of the endogenous potassium was lost from the mitochondria.

A test was made in order to find out whether any transformation of citrate occurred in the presence of mitochondria at 0°. Two samples of the mitochondrial suspension in 0·25M-sucrose (2 ml., containing 50 mg. of M) were added to equal volumes of sodium citrate, so that the final concentration of citrate was 10 mM. One sample was deproteinized immediately with 1 ml. of 30% (w/v) trichloroacetic acid, and the other after 75 min. at 0°. When the centrifuged extracts were analysed, they contained the same amounts of orthophosphate and organic phosphate, but the second sample contained 4% less citrate than the first, probably owing to the small residual activity of aconitase at 0°. This small disappearance of citrate does not materially affect the distribution ratios.

Distribution of sodium orthophosphate between mitochondrial pellets and medium. Labelled polyglucose was omitted from the medium in this experiment (for reasons of economy), so the concentrations of solutes were calculated on the basis of the total water content of the mitochondrial pellets. The pellets centrifuged from 0·25M-sucrose (Table 4, Expt. 1) contained 9·7 millimolal orthophosphate and 26·7 millimolal organic phosphate. At the same time as the centrifuge was started, a sample (2 ml.) of the stock suspension of mitochondria in 0·25M-sucrose was deproteinized by adding 1 ml. of 30% (w/v) trichloroacetic acid. The centrifuged extract was analysed for total phosphate and orthophosphate. When the results were compared with the analyses of the mitochondria which had been centrifuged before deproteinizing, it was found that the orthophosphate content of the centrifuged mitochondria was 10% too high. Apparently about 3% of the endogenous organic phosphate of the mitochondria had hydrolysed during the centrifuging and subsequent manipulations at 0° (see Methods) before trichloroacetic acid could be added to the pellets. Yet in the preceding experiment (testing for disappearance of citrate) mitochondria maintained in suspension at 0° showed no changes in the ratio of organic phosphate and orthophosphate. As the organic-phosphate content of the mitochondrial pellets was

the same in each experiment in Table 4, it was assumed that the hydrolysis was the same throughout, for the purpose of calculating the distribution ratios for orthophosphate added to the suspension.

When mM-sodium phosphate was added to the medium (Expt. 2) both ions were appreciably adsorbed by the mitochondrial pellet, the degrees of adsorption being 3·2 for sodium and 4·2 for orthophosphate. With an external concentration of 10 mM-sodium phosphate, the concentrations in the pellet were still considerably higher than those in the medium (Expt. 3). Resuspending the mitochondria in 89 mM-sodium phosphate and recentrifuging (Expt. 4) caused a large increase (73 %) in the water content of the mitochondrial pellet, compared with that centrifuged from 0·25 M-sucrose. This was the only occasion in the present work or in that previously reported (Amoore & Bartley, 1958) in which the water content of mitochondria or mitochondrial pellets increased by more than 25 % as a result of manipulations and changes of suspension medium carried out at 0°.

The concentration of sodium in the swollen mitochondrial pellet (143 millimolal) was nearly as high as that in the medium (147 millimolal). The sodium space of the pellet was 97 % and the orthophosphate space 82 %, compared with the sucrose space (Expt. 1) of 70 %. The sucrose space of the pellet in Expt. 4 was 69 %. Apparently the mitochondria are considerably more permeable to phosphate than to sucrose. The swelling observed in Expt. 4 was accompanied by the loss of 58 % of the endogenous potassium of the mitochondria, but no loss of organic phosphate.

Distribution of sodium adenosine 5'-phosphate between mitochondria and medium. The concentration of endogenous organic phosphate in the mitochondria was apparently 47·9 millimolal (Table 5, Expt. 1), which is high compared with the concentrations of AMP added to the medium in Expts. 2 and 3 (1 and 10 mM respectively). The endogenous content must be accurately known, because the analyses for organic phosphate were used to estimate the distribution of added AMP. However, a sample of the mitochondrial suspension in 0·25 M-sucrose,

deproteinized without prior centrifuging, showed that about 13 % of the endogenous organic phosphate had hydrolysed during the centrifuging and subsequent manipulation. The extent of the hydrolysis was not constant, for in the presence of AMP (Expts. 2–4) the concentration of orthophosphate in the mitochondria was much less than that found in Expt. 1. In these circumstances the calculated distribution ratios for AMP lack precision, particularly in Expts. 2 and 3.

Adding mM-sodium AMP to the medium (Expt. 2) had no effect on the organic-phosphate content of the mitochondria. Whereas there was apparently no adsorption of AMP, there was a small uptake of sodium, the degree of adsorption being 1·4. Raising the AMP concentration in the medium to 10 mM (Expt. 3) had very little effect on the concentration of organic phosphate in the mitochondria, but the internal sodium concentration was again rather greater than the external. Even resuspending the mitochondria in 85 mM-sodium AMP (Expt. 4) raised the organic phosphate concentration in the mitochondria from 47·9 to only 56·5 millimolal. The calculated sodium space was only 25 %, compared with the sucrose space of 45 % (Expt. 1). Thus the mitochondria were much less permeable to AMP than they were to sucrose. The low permeability to AMP was confirmed in a later experiment (Table 7), in which the breakdown of endogenous organic phosphates was prevented. In Expt. 4, 23 % of the endogenous potassium of the mitochondria was lost.

Distribution of sodium adenosine triphosphate between mitochondria and medium. An attempt was made to limit the breakdown of labile organic phosphates by centrifuging the mitochondria through silicone fluid, according to the procedure of Werkheiser & Bartley (1957), and placing the tubes in a bath of ethanol–solid CO_2 as soon as the centrifuge stopped. In this procedure the mitochondrial pellets remained frozen until they were deproteinized with trichloroacetic acid, thereby avoiding the period of 20–30 min. at 0°, which elapsed in the experiments described above,

Table 5. *Distribution of sodium adenosine 5'-phosphate between mitochondria and medium*

Details are as given in Table 3, except that 85 mM-sodium adenosine 5'-phosphate was used instead of sodium citrate. Analysis of the 85 mM-sodium adenosine 5'-phosphate gave 137 mM-sodium, 75·5 mM-organic phosphate and 0·2 mM-orthophosphate.

Expt. no.	Internal water content of mitochondria (l./kg. of M)	Concn. of solutes (millimolal)							
		Organic phosphate		Sodium		Sucrose		Potassium. Internal	Orthophosphate. Internal
		External	Internal	External	Internal	External	Internal		
1	1·61	0·2	47·9	<0·1	0·9	235	106	70·5	20·0
2	1·68	1·2	45·2	1·8	3·4	234	114	68·4	14·2
3	1·70	9·8	48·7	16·2	18·6	210	103	65·6	13·5
4	1·93	66·9	56·5	124	64·5	18·1	11·5	45·4	14·1

Table 6. *Distribution of sodium adenosine triphosphate between mitochondria and medium*

In Expts. 1–3, a suspension of mitochondria in 0·25 M-sucrose (2·5 ml.) was added to the appropriate volume of 51 mM- sodium adenosine triphosphate. In Expt. 4, 2·5 ml. of the original suspension in 0·25 M-sucrose was centrifuged, and the mitochondria were resuspended in 2·5 ml. of 51 mM-sodium adenosine triphosphate. Labelled polyglucose (0·25 ml. of 10%, w/v) was added, and 2 ml. of the mixtures was layered over 2·5 ml. of silicone fluid (no lower layer of 'fixative' was used). After 18 min. at 0° in contact with ATP, the mitochondria were centrifuged at 25 000 g for 2·5 min., and then frozen in a bath of ethanol–solid CO_2. Analysis of the 51 mM-sodium adenosine triphosphate gave 200 mM-sodium, 159 mM-organic phosphate and 0·6 mM-orthophosphate.

Expt. no.	Internal water content of mitochondria (l./kg. of M)	Concn. of solutes (millimolal)								
		Organic phosphate		Sodium		Sucrose		Potassium.	Orthophosphate	
		External	Internal	External	Internal	External	Internal	Internal	External	Internal
1	1·79	0·2	38·7	0·1	4·0	226	153	70·0	0·1	17·9
2	1·84	2·2	39·0	2·8	10·2	226	138	69·8	0·7	23·5
3	1·66	27·6	41·9	35·0	36·8	186	106	73·1	4·2	37·5
4	1·90	122	44·6	166	88·0	16·2	9·7	49·7	13·8	62·1

between centrifuging and deproteinizing. The period of centrifuging through silicone fluid was shorter (2·5 min.) than that required without silicone (10 min.). Despite these precautions, comparison of the analyses of the centrifuged mitochondria (Table 6, Expt. 1) with a sample of the stock suspension directly deproteinized showed that about 11% of the organic phosphate of the mitochondria had hydrolysed. This breakdown must have occurred while the centrifuge was running.

The added ATP also underwent considerable dephosphorylation, as shown by the concentrations of orthophosphate found in the supernatant fluid (Table 6, Expts. 2–4; less than 0·4% of the phosphate in the 51 mM-sodium ATP solution was inorganic). This dephosphorylation must have occurred during the 18 min. at 0° before the mitochondria were centrifuged out of the medium. The reaction mixture contained 20 mg. of M/ml. A Lineweaver & Burk (1934) reciprocal plot of the data gave a straight line, from which it was calculated that the maximum velocity of the dephosphorylation at 0° ($V_{max.}$) was 2·5 μmoles of orthophosphate liberated/mg. of M/hr., and the Michaelis constant (K_s) was 26 mM-ATP.

The concentrations of orthophosphate found in the mitochondria (Table 6, Expts. 2–4) were disproportionately high compared with the external concentrations, considering the distribution of orthophosphate observed in Table 4. Evidently dephosphorylation of ATP must have continued in the mitochondrial pellet during the centrifuging and until the pellet was frozen. The unexpectedly high activity of the mitochondrial 'adenosine triphosphatase' at 0° nullified the main purpose of the experiment, which was to determine the permeability of the mitochondria to ATP. However, the presence of ATP in the medium caused very slight increases in the organic phosphate content of the mitochondria (Expts. 2–4), which showed that

there was a small degree of permeability or adsorption.

In Expt. 3, 10 mM-ATP in the medium produced a 7% decrease in the water content of the mitochondria. Resuspending the mitochondria in 51 mM-sodium ATP (Expt. 4) caused the loss of 24% of the endogenous potassium. In Expt. 2 the degree of adsorption of sodium was 2·2. The sodium space of the mitochondria was 51% (Expt. 4) compared with the sucrose space of 68% (Expt. 1).

Distribution of adenosine 5'-phosphate and adenosine triphosphate, measured after short contact and rapid fixation. This experiment employed the technique of Werkheiser & Bartley (1957), in which the mitochondria are deproteinized by centrifuging them through silicone fluid into a lower layer of formalin–perchloric acid 'fixative'. Two samples of each suspension of mitochondria were centrifuged simultaneously through silicone fluid, one with and the other without fixative (see Table 7 for details). The amounts of orthophosphate and of organic phosphate found in the mitochondria, centrifuged from 0·25 M-sucrose and fixed by this method, agreed well with the amounts present in a sample of the suspension of mitochondria in 0·25 M-sucrose which had been deproteinized without centrifuging. However, the mitochondrial pellet centrifuged into the fixative contained as little as 70% of certain solutes as compared with the pellet centrifuged without fixative (compare Werkheiser & Bartley, 1957). The deficiency was greater with external solutes than with endogenous solutes of the mitochondria. Conversely, there was an excessive amount of labelled polyglucose. Apparently some fluid passes upwards through the silicone layer after the downward passage of the mitochondrial pellet, but the details of the process are obscure. In view of these difficulties, the analyses of the mitochondrial pellet fixed in formalin–perchloric

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Table 7. *Distribution of adenosine 5'-phosphate and adenosine triphosphate,
measured after short contact and rapid fixation*

A concentrated suspension of mitochondria in 0·25M-sucrose (1 ml.) was added to (Expt. 1) 4 ml. of 0·25M-sucrose, (Expt. 2) 4 ml. of 85 mM-sodium adenosine 5'-phosphate, and (Expt. 3) 4 ml. of 51 mM-sodium adenosine triphosphate. Each tube also contained 0·5 ml. of 10% (w/v) labelled polyglucose. Two samples (2 ml.) of each mixture were immediately layered over (a) 2·5 ml. of silicone fluid and (b) 1·5 ml. of silicone fluid above 1 ml. of formalin–HClO₄ 'fixative'. After contact with the AMP for 3·25 min., and with the ATP for 1·25 min., the mitochondria were centrifuged at 25 000 g for 2·5 min. and then frozen. Analysis of the 85 mM-sodium adenosine 5'-phosphate gave 153 mM-sodium, 89·1 mM-organic phosphate and 0·1 mM-orthophosphate; the 51 mM-sodium adenosine triphosphate gave 186 mM-sodium, 151 mM-organic phosphate and 0·8 mM-orthophosphate.

Expt. no.	Internal water content of mitochondria (l./kg. of M)	Concn. of solutes (millimolal)								
		Organic phosphate		Sodium		Sucrose		Potassium.	Orthophosphate	
		External	Internal	External	Internal	External	Internal	Internal	External	Internal
1	1·62	0·2	59·0	0·1	2·4	219	136	71·0	<0·1	11·0
2	1·71	64·2	77·0	110	64·9	38·6	21·2	67·9	0·1	11·0
3	1·84	97·8	68·6	137	65·1	38·9	25·5	63·6	12·4	24·3

acid were used only to determine the ratio of orthophosphate and organic phosphate. This ratio was then used in order to assign to its components the total phosphate found in the pellet centrifuged without fixative.

The scope of the experiments in Table 7 was limited to the use of high concentrations of AMP (Expt. 2) and ATP (Expt. 3), in order to determine the permeability of the mitochondria to these solutes. (For reasons given earlier, attempts to detect adsorption of AMP and ATP by means of phosphate analyses are unlikely to yield conclusive results.) The time of contact of the mitochondria with ATP was decreased to 1·25 min. in an attempt to minimize the liberation of orthophosphate.

In agreement with the results of Werkheiser & Bartley (1957), 84% of the acid-extractable phosphate of the mitochondria sedimented from 0·25M-sucrose at 0° was organic phosphate (Expt. 1). The sucrose space of the mitochondria was 62%. In Expt. 2 the sodium space was 57% and the AMP space was 33%. The concentration of orthophosphate in the mitochondria was unaffected by the presence of AMP.

In Expt. 3 the inclusion of a high concentration of organic phosphate (ATP) in the medium had little effect on the organic phosphate concentration in the mitochondria. The sodium space was 46% and the ATP space was only 17%. Thus the permeability of the mitochondria at 0° to AMP was about half that to sucrose, and the permeability to ATP was only a little over one-quarter of that to sucrose.

Although in Table 7, Expt. 3, the ATP remained in contact with the mitochondria at 0° for less than one-tenth of the time allowed in Table 6, Expt. 4, before centrifuging, nearly as high a concentration of orthophosphate (12·4 millimolal) appeared in the supernatant fluid. (A lower concentration of mito-

chondria, 17 mg. of M/ml., was present in the experiments of Table 7.) The increment in internal-orthophosphate concentration in Expt. 3 (Table 7) compared with Expts. 1 and 2 was 13·3 millimolal, which was approximately the same as the external concentration of orthophosphate which developed. Thus the uptake of orthophosphate by the mitochondria in the presence of ATP was rather less than the uptake observed with a comparable concentration of orthophosphate alone (Table 4, Expt. 3).

There were no changes in the endogenous potassium content of the mitochondria in the experiments in Table 7. The brief time of exposure to the new medium before centrifuging (1·25 min. in Expt. 3) nearly allowed the sucrose to become re-equilibrated, for the sucrose space in Expt. 3 was 66% compared with 62% in Expt. 1. This was in agreement with the conclusion reported earlier (Amoore & Bartley, 1958) that part of the intra-mitochondrial volume reaches equilibrium very rapidly with the medium.

DISCUSSION

Interpretation of studies on the distribution of solutes. The object of measuring the distribution of solutes between mitochondria and medium was to determine the permeability of the mitochondria. However, several factors tended to complicate the interpretation of the results. These were as follows: endogenous solute content; adsorption of solutes; enzymic transformation of solutes; disparity between sodium and anion spaces; and apparent non-conformity with osmotic considerations. These difficulties, which were of varying importance for each anion, will be discussed separately.

The concentrations of endogenous solutes were small compared with the highest concentrations added to the medium (Expts. 4 of each table),

except in the study of the organic phosphates. Here the conclusions about the permeability of the mitochondria depend upon the assumption that the endogenous organic phosphates remain within the mitochondria and fail to exchange with phosphates in the medium. If any exchange of organic phosphates occurred, the permeability indicated by the solute spaces would be too low.

When appreciable degrees of adsorption of the added solute were shown with the lowest external concentration (as in Expt. 2 of Tables 2–4), it followed that the apparent permeabilities indicated by the solute spaces were too high. This was the situation with succinate, citrate and orthophosphate. With the available data it was not possible to determine how much of the measured space represented adsorption.

Enzymic transformation of added solutes was assessed at 0° for citrate, orthophosphate, AMP and ATP. With orthophosphate and AMP there was no esterification or hydrolysis. A very small disappearance of citrate occurred, possibly by aconitase activity, but this did not materially affect the conclusions. Hydrolysis of ATP was considerable during brief contact with the mitochondria at 0° (Tables 6 and 7). It has often been reported (e.g. by Maley & Johnson, 1957) that fresh rat-liver mitochondria prepared in 0·25M-sucrose have little or no adenosine triphosphatase activity. The adenosine triphosphatase activity found in the present work at 0° (Table 6) was roughly four times as great as that found by Maley & Johnson at 23°, and an even greater rate was noted in Table 7.

Disparity between the sodium and anion spaces of the mitochondria was observed with every salt tested. The sodium space was always substantially larger than the anion space. On exposing the mitochondria to high salt concentrations, especially sodium citrate or sodium orthophosphate, there were considerable losses of potassium from the mitochondria, with little or no loss of organic phosphates. An exchange of mitochondrial potassium for sodium of the medium accounted for some of the excess of the sodium space over the anion space, but in several experiments (e.g. Table 7) little or no loss of potassium occurred. Presumably the electrical neutrality of the mitochondria was maintained by exchanges of ions not estimated in the present study.

The osmotic behaviour of the mitochondria was apparently irregular in some experiments. It was expected that transferring the mitochondria from 0·25M-sucrose into a solution (with the same osmolarity) of another solute, to which they were more permeable, would result in swelling of the mitochondria, and vice versa. The first expectation was realized with sodium orthophosphate, to which the mitochondria were more permeable than they were to sucrose. A large increase in the water content of the mitochondrial pellet was observed on transfer to the saline medium (Table 4, Expt. 4). However, the converse was not found with sodium AMP or sodium ATP, to which the mitochondria were much less permeable than they were to sucrose. Instead of the expected shrinkages of the mitochondria, small increases took place in their internal water content (Table 7, Expts. 2 and 3). This result is not understood; it was not due to retention in the mitochondria of sucrose, which became redistributed in accordance with the sucrose space found in Expt. 1. These findings suggest that inferences about the permeability of subcellular particles, derived from observations of their osmotic behaviour alone (e.g., Cleland, 1952; Tedeschi & Harris, 1955), require confirmation by measurements of solute distributions.

Metabolic implications of the results. The present work, together with earlier studies (Werkheiser & Bartley, 1957; Amoore & Bartley, 1958; Bartley & Amoore, 1958), has provided information about the properties of mitochondria at 0°. The evidence was obtained by direct chemical analysis, in the virtual absence of metabolic activity of the mitochondria. It has become apparent that at 0° the mitochondria are rather impermeable to a variety of solutes of metabolic importance. Furthermore, the mitochondria are capable of adsorbing considerable quantities of ions from dilute solution at 0°. These data are intended to provide a basis for further studies on the effect of temperature on the permeability and adsorbency of the mitochondria, and on the possible contribution of active transport when metabolism is in progress.

The metabolic significance of the limited permeability of mitochondria, in relation to the intracellular distribution of enzymes, has been discussed by Schneider & Hogeboom (1956) and Hogeboom, Kuff & Schneider (1957). However, some of the reported distributions of enzymes may require revision, because the relative impermeability of the mitochondrial membrane to the added substrate may have prevented the intramitochondrial enzyme from exhibiting its full activity in the assay. This probably happened with mitochondrial aconitase (Dickman & Speyer, 1954). In certain metabolic pathways, it appears to be necessary for intermediates to pass from the soluble fraction of the cell to the mitochondrial fraction, or vice versa. Thus citrate is formed almost exclusively by the mitochondria (Kalnitsky, 1949), whereas most of the *iso*citric dehydrogenase is in the soluble fraction (Hogeboom & Schneider, 1950; see, however, Ernster & Lindberg, 1958). Experiments in which cell fractions were recombined in various ways have shown that for oxidation to proceed at a maximum rate there must be co-operation between

the particulate and soluble fractions, as Siekevitz (1952) found with α-oxoglutarate oxidation. The permeability of the mitochondria may therefore play an important role *in vivo* in controlling the overall rate of metabolism.

SUMMARY

1. The distribution of solutes between isolated rat-liver mitochondria and the suspension medium at 0° was studied by centrifuging and analysing the sedimented pellet and supernatant fluid. The distributions of the sodium salts of pyruvate, succinate, citrate, phosphate, adenosine 5′-phosphate and adenosine triphosphate were measured over a wide range of external concentrations. The volume of extraparticulate fluid in the pellet was estimated, and the distribution of sucrose was also measured.

2. With mM salt in the medium, marked uptakes of succinate, citrate and phosphate by the mitochondria were observed. The increases in concentration of these anions in the mitochondria were four to ten times the concentrations in the medium.

3. About two-thirds of the mitochondrial water was accessible to sucrose, and the space accessible to pyruvate, succinate and citrate was of the same order. The mitochondria were rather more accessible to phosphate than they were to sucrose. They were much less accessible to adenosine 5′-phosphate and to adenosine triphosphate, the permeability to the latter being one-quarter of that to sucrose.

4. With each salt, the penetration of sodium was somewhat greater than the penetration of the anion. Part of the difference may be due to the loss of endogenous potassium from the mitochondria.

5. The changes in water content of the mitochondria when they were suspended in solutions of the sodium adenosine phosphates were not consistent with osmotic considerations based on the measured distribution of solutes.

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