The Study of Steady-State Concentrations of Internal Solutes of Mitochondria by Rapid Centrifugal Transfer to a Fixation Medium

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Reliable measurements of the distribution of solutes between mitochondria and their surrounding medium depends ideally on the rapid isolation of mitochondria from their medium. Moreover, it is essential that the solute composition of mitochondria should not change during this process. A method is described in this paper which deals with this problem. The mitochondria are centrifuged through a layer of silicone fluid immiscible with water, of a density intermediate between that of the particles and that of the medium. A high-density aqueous 'fixative' beneath the silicone can be used to stabilize labile compounds. Particles centrifuged in this manner carry with them an adherent film of their incubation medium. The amount of this adherent fluid can be measured by the inclusion of a radioactive high molecular (20 000–50 000) polysaccharide, such as inulin or polyglucose.

This paper describes the elaboration of this technique and its application to the study of the solutes of rat-liver mitochondria.

MATERIALS AND METHODS

Special chemicals

Silicone Fluid 702 [Edwards and Co., (London) Ltd.], sp.gr. 1·085, and Silicone Fluid M.S. 200/10 cs. (Hopkins and Williams Ltd.), sp.gr. 0·94, were mixed in suitable proportions to yield fluids of the required density. Silicone Fluid M.S. 1107 was prepared as a 2% (v/v) solution in CCl_4. Glassware to be rendered water-repellent was dipped in this solution, drained and heated at 110° for 2 hr.

Sodium adenosine triphosphate (ATP) was a commercial product (Zellstoff-fabrik Waldhof, Pharmazeutische Abteilung, Wiesbaden).

[^{14}C]Carboxypolyglucose (later referred to as 'labelled polyglucose') and [^{14}C]carboxyinulin were prepared by the following procedure based on the general method described by Isbell (1953). To a mixture of 1 m-mole of polymeric sugar and 1 m-mole of $NaHCO_3$ was added 1 m-mole of $K^{14}CN$ and 1 m-mole of KOH in a volume of 5 ml. or more if necessary. The mixture was held at room temperature until the reaction was complete. With polyglucose the time required was about 24 hr. whereas with inulin 3 days were necessary. The solution was heated in a water bath for 4–6 hr. at 80°, water being replaced as it was lost, and then dialysed overnight against water. In the preparation of polyglucose, 0·056 m-mole of each reagent, and in that of inulin, 0·014 m-mole, were used. No carrier KCN was used. (Polyglucose was presented by Dr P. T. Mora, National Cancer Institute, National Institutes of Health, Bethesda, U.S.A.) All other chemicals were analytical grade when available.

Homogenization media

Sucrose (0·25 M). In early studies sucrose was dissolved in the laboratory distilled water, chilled, and used directly. The pH of this solution was variable, but usually acid (pH 5–6) because of dissolved CO_2. The percentage dry wt. (see below) of mitochondria prepared in this acid sucrose was usually between 21 and 27 and they appeared visibly swollen when viewed in the phase-contrast microscope. Removal of most of the CO_2 from the water was accomplished by boiling for 10 min. in an open beaker, by prolonged evacuation on a water pump or by bubbling CO_2-free air through it for 2–3 hr. Sucrose was then dissolved in this water, the pH adjusted to 7·8 with aminotrishydroxymethylmethane (0·2 M) and the solution stored at 2° for not longer than 48 hr. The percentage dry wt. of mitochondria prepared in this medium was 31–34 and these mitochondria appeared quite dark and unswollen.

Modified sucrose solutions. Solutions of sucrose (0·25 M)–disodium ethylenediaminetetra-acetate (EDTA) (0·001 M), sucrose (0·25 M)–polyvinylpyrrolidone (PVP) (7·3%, w/v) and sucrose (0·25 M)–dextran (6%, w/v) were prepared in CO_2-free water and adjusted to pH 7·8 in a similar manner. PVP (L. Light and Co., Ltd.) was used as obtained or was dialysed exhaustively against distilled water. Dextran (gifts from Glaxo Laboratories Ltd., and Benger's Laboratories Ltd.) was used as received or after exhaustive dialysis against distilled water.

Mitochondrial fractionation

Sucrose mitochondria. These were prepared by the method of Schneider (1948) with minor modifications as suggested by the work of Appelmans, Wattiaux & Duve (1955). The complete fractionation procedure was performed at 0–2°. Chilled rat liver was homogenized in a stainless-steel Potter–Elvejhem homogenizer in 4 vol. of cold 0·25 M-sucrose solution and centrifuged for 10 min. at 2000 rev./min. (600 *g*) in rotor 822 of the refrigerated International Centrifuge (PR 2). The residue was resuspended in 1·5 vol. of sucrose and centrifuged for 10 min. at 1500 rev./min. (340 *g*). The combined supernatants were then centrifuged

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at full speed (4200 rev./min., 2700 *g*) for 20 min. The mitochondrial residue thus obtained was washed twice by resuspension in 1·5 vol. of sucrose and centrifuging for 10 min. in the 4-place high-speed head of the centrifuge at 2400 rev./min. (8500 *g*). All pinkish, freely flowing residue (fluffy layer) was discarded along with the supernatant in each of these decantations. It is worth emphasizing at this point that a voluminous fluffy layer appears only on washing the 'mitochondrial' sediment with pure sucrose solution. The sedimentation properties of both the true mitochondria and the fluffy layer are so modified in this medium that higher centrifugal forces are required to obtain a well-packed sediment. The presence of substances such as EDTA, PVP and dextran as well as 'supernatant' (see below) in the washing fluid greatly reduces the amount of fluffy layer and preserves the original sedimentation properties.

The final mitochondrial residue was resuspended in a small volume of sucrose and centrifuged at the full speed of the centrifuge (4200 rev./min. 26000 *g*) for 5 min. The supernatant was decanted, the tube well drained and the firmly packed residue was then resuspended in the desired incubation medium. The yield in a typical experiment was 4·4 g. wet wt. (1·5 g. dry wt.) of mitochondria/100 g. of liver.

Sucrose–EDTA mitochondria. The sucrose–EDTA medium was used in this preparation and the fractionation procedure outlined above was followed with one exception. The mitochondrial washings were performed in the large rotor 822 at full speed as in the first sedimentation of the mitochondria. As noted above, it was observed that in the presence of EDTA the usual fluffy layer did not form to any appreciable extent during washing, and lower centrifugal forces were needed to sediment the particles. During the washing procedure two distinct layers were observed in the sediment. The upper, somewhat pinkish layer was rather gelatinous and on gentle swirling could be poured off as an intact mass, leaving the buff-coloured mitochondrial layer behind.

Sucrose–PVP mitochondria and sucrose–dextran mitochondria. The preparation was as for sucrose–EDTA mitochondria.

'*Sucrose-supernatant mitochondria*'. The supernatant from the first mitochondrial centrifuging of a sucrose homogenate was further centrifuged for 1 hr. at 105000 *g* (30000 rev./min. in rotor 40) in the Spinco preparative ultracentrifuge, to remove remaining particles. The supernatant was stored in the deep freeze. 'Sucrose-supernatant mitochondria' were obtained by preparing sucrose mitochondria through the stage of the first sedimentation of the mitochondria. Washing of the mitochondria was then accomplished by the method for sucrose–EDTA mitochondria with 'supernatant' used as the washing medium.

Incubation conditions

The final mitochondrial pellet was resuspended in the chosen suspension medium at 0°. Labelled polyglucose [0·1 vol. of 9% (w/v) solution] was added plus any other desired components. The suspension was allowed to stand at 0° for at least 5 min. before the mitochondria were separated from their suspending medium by the centrifuging procedure.

Analytical methods

Chemical methods. Sodium and potassium were measured with a Beckman Flame Photometer, Model 9200. All phosphate determinations were made by the method of Berenblum & Chain (1938) as modified by Bartley (1953). Wet-ashing was performed by the method of Hanes & Isherwood (1949) with the addition of a drop of 5% (w/v) ammonium molybdate to facilitate the combustion. Sucrose was determined initially by the method of Bacon & Bell (1948), but later the method of Kulka (1956) was used, since it gave the more consistent results claimed by the author.

Chloride was determined by the method of Sanderson (1952). The Ag electrode was cleaned before use by brief immersion in 6N-HNO_3. With this cleaned electrode 0·1N-HNO_3 was used as the titration medium since the meter response was more rapid with this medium than with acetic acid.

Radioactivity determinations

Planchets for measurement of radioactivity were prepared on lens-tissue circles (16 mm.) centred on circular cover slips (19 mm.). Solutions containing trichloroacetic acid (TCA) were pipetted (0·05 ml.) on to planchets previously wetted with 0·01–0·02 ml. of a 10% (w/v) solution of mannitol in water. Solutions containing $HClO_4$ were pipetted (0·05 ml.) on to planchets which already contained 0·15 ml. of 0·4M-NaOH containing bromocresol purple. The liquid was stirred with a gentle stream of air until the colour of the indicator was uniformly purple. All planchets were dried at 60° in a small oven placed in a fume cupboard and then counted under a mica end-window counting tube. A minimum of 2500 counts were accumulated, which represents a standard error of less than 2%.

EXPERIMENTAL AND RESULTS

Whittam & Davies (1954) observed that the turnover of cellular sodium and potassium cannot be described by one rate constant. This observation suggests that more than one diffusion barrier exists in tissues and it has been thought that the mitochondrial membrane may represent such a second barrier. Bartley & Davies (1954), MacFarlane & Spencer (1953) and Stanbury & Mudge (1953) have in fact demonstrated concentration gradients between mitochondria and medium for several ions, dependent in at least one case (Bartley & Davies, 1954) on metabolism for their maintenance. In this work mitochondria had been separated for analysis by centrifuging, decantation and addition of acid to the pellet. It was recognized by Bartley & Davies (1954) that changes in the mitochondrial content of these labile compounds might occur in the time between sedimentation and addition of acid, but no way of avoiding this difficulty was then available. This problem does not occur in experiments with tissue slices, since the slices may be easily lifted out of their medium with forceps, blotted, and transferred to acid in a matter of seconds. The minimum time for the isolation of mitochondria by centrifuging, on the other hand, is a matter of minutes. This interval is indeed significant, for preliminary experiments showed that 19% of the endogenous organic phosphate of a mitochondrial suspension

Table 1. *Comparison of extraction of phosphate from mitochondria by various fluids*

To 1 ml. of the extracting fluid at 0° was added 1 ml. of a mitochondrial suspension in 0·25 M-sucrose containing 0·04 M-NaCl and 0·04 M-KCl. The extracting fluids contained the components listed in columns 1 and 2. The concentrations were such that the mixture with the mitochondria was of density 1·06 and contained either 0·5 M-$HClO_4$ or 10% (w/v) TCA. The phosphate is given as a percentage of that extracted by 10% (w/v) TCA alone.

Fixing acid	Density adjuster	Orthophosphate (%)	Organic phosphate (%)
TCA	Ammonium trichloroacetate	116	107
TCA	Urea	108, 101	105, 98·5
TCA	Formalin	94·5	98·8
$HClO_4$	Ammonium perchlorate	104	114
$HClO_4$	Urea	101	107
$HClO_4$	Formalin	96·5	97·7

sedimented at room temperature was hydrolysed in this brief time (about 5 min.). We have therefore developed a procedure for essentially simultaneous separation and 'fixation' by centrifuging mitochondria through a layer of silicone fluid immiscible with water into an aqueous acid 'fixative'. This acid must be of such a strength and nature that enzymic activities are very rapidly stopped, all unprecipitated solutes are extracted and the labile components of the extract are stabilized. For convenience we propose to call this process 'fixation' and the extracting fluid a 'fixative'. By this method the breakdown of organic phosphate before fixation was eliminated.

Treatment of centrifuge tubes. When two aqueous layers in a centrifuge tube are separated by a silicone layer, there is a tendency for the aqueous layers to become continuous down the walls of the tube, owing to the preferential wetting of the glass by the water. This was prevented by treatment of the tubes with M.S. 1107 silicone preparation, as described under Materials and Methods. The tubes then became water-repellent and complete isolation of the upper and lower water layers was achieved.

Choice of silicone density. A density differential of 0·015 between the mitochondrial suspending fluid and the silicone fluid was maintained. In addition, the overall density of the mitochondrial suspension was kept at least 0·007 below that of the silicone. Such a suspension contains not more than 25 mg. of dry mitochondria/ml.

Choice of fixative. The fixative must of course be of a density greater than the silicone above it. To achieve this density with conventional fixatives requires a concentration much greater than normally used [e.g. 20–30% (w/v) TCA or 2 M-$HClO_4$]. Two difficulties arise with the use of these reagents. TCA is extremely soluble in silicone fluids as well as water. In some manner not fully understood this results in a transfer of appreciable quantities of TCA and extracted mitochondrial contents to the fluid above the silicone upon centrifuging. Perchloric acid (2 M), although totally insoluble in silicone fluid, extracts more organic phosphate (about 16%) from the mitochondria than the generally accepted extracting fluids [e.g. 10% (w/v) TCA] and is thus also unsatisfactory. Search was therefore made for substances which would increase the density of the conventional extracting fluids [10% (w/v) TCA or 0·5 M-$HClO_4$]. Such materials should not increase the extraction of organic phosphates or be soluble in silicone, and ideally should not interfere with the subsequent analytical procedures. Table 1 summarizes the tests on various extraction fluids compared with 10% (w/v) TCA. On the basis of these results the ammonium salt mixtures were rejected.

The formalin–$HClO_4$ mixture (density 1·10) was used in the subsequent studies. The presence of so reactive a substance as formalin in the fixative is an obvious disadvantage as it may interfere with many analytical procedures (e.g. for sucrose or keto acids). Search for an inert substitute for formalin is proceeding.

Centrifuging procedure and recovery of mitochondrial phosphate in the extracting fluid. For separation of the mitochondria from their supernatant medium, the 6-place high-speed head of the centrifuge was used. The tubes were thick-walled (2 mm.) Pyrex glass of capacity 5·5 ml. A volume (1 ml.) of the selected fixative (when used) was pipetted into the bottom of the centrifuge tube, care being taken not to leave adherent drops on the wall. Silicone (1·5 ml., or 2·5 ml. when the fixative was omitted) of the appropriate density was layered over the fixative by means of a pipette whose tip was bent at a right angle. Finally 2 ml. of the mitochondrial suspension at 0° was added in a similar manner.

In preliminary experiments the control handle of the centrifuge was turned five divisions every 6 sec., reaching maximum in 1 min. Under these conditions (Table 2, line 1) a variable amount of the mitochondrial phosphate was found in the supernatant. Table 2 shows the effect of variation in the centrifuging procedure. Procedure 5 with the formalin–$HClO_4$ mixture (sp.gr. 1·10) was the most satisfactory in that the amount of mitochondrial

Table 2. *Influence of centrifuging procedure on the contamination of the supernatant fluid by mitochondrial phosphate*

The centrifuge tubes contained 2 ml. of mitochondrial suspension at 0° in 0·25 M-sucrose containing 0·04 M-KCl and 0·04 M-NaCl layered over 2·5 ml. of silicone or over 1·5 ml. of silicone beneath which was 1 ml. of fixative [containing either 0·5 M-$HClO_4$ or 10 % (w/v) TCA]. The density of the fixative was adjusted to the indicated values with TCA, urea or formalin. The centrifuge was switched off after 2·5 min. running time. After centrifuging the supernatant was carefully stirred and a sample withdrawn for analysis of its phosphate content. Each number is the excess (μmoles) found in the tube with fixative over that found in the tube with silicone alone.

Nature of acid fixative	TCA					$HClO_4$				
Density adjuster	TCA	Urea		Formalin		Urea	Formalin			
Sp.gr.	1·15	1·08	1·10	1·06	1·13	1·06	1·06	1·08	1·10	1·11
Centrifuging procedure										
1. Handle turned 5 div. every 6 sec.	1·46	—	—	0·97	—	0·55	0·27	—	—	—
2. Handle turned 5 div. every 12 sec.	—	0·24	0·33	0·81	—	0·52	0·39	0·23	0·13	—
3. Handle turned in such a manner that the rate of increase of angular velocity was constant, reaching the maximum in 2 min.	—	0·32	0·46	—	—	—	—	0·06	0·09	—
4. Handle turned in such a manner that the rate of increase of centrifugal force was constant, reaching the maximum in 2 min.	—	0·33	0·49	0·41	0·19	—	—	0·16	0·11, 0·08	0·33
5. Handle turned to maximum as rapidly as possible	—	—	—	—	0·09	—	—	—	0·07, 0·02	0·20

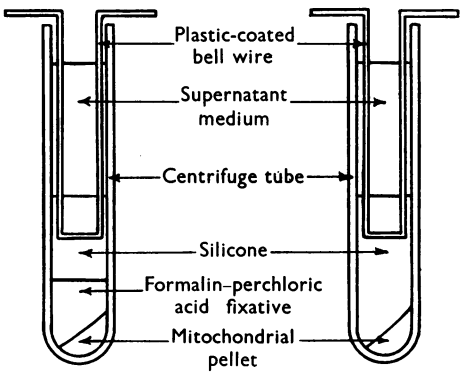

Fig. 1. Centrifuge tubes containing mitochondria sedimented through silicone fluid.

phosphate contaminating the supernatant in this case was less than 3 % of the total acid-extractable phosphate. Table 2 also shows the very large amount of mitochondrial phosphate (approx. 50 %) which can be transferred to the supernatant when concentrated TCA solutions are used as fixatives.

Although nearly all the mitochondrial phosphate was recovered in the formalin–$HClO_4$ extract under these conditions, varying amounts of the mitochondrial sodium, potassium and chloride were still transferred to the supernatant medium during centrifuging. Estimation of these latter stable ions was therefore made on pellets sedimented through silicone alone.

The solubility of TCA in silicone fluids suggested that this and other substances, dissolved in silicone, might serve as fixatives without any need for an aqueous layer. TCA, I_2 and SO_2 solutions in silicone were tested. All fixed the mitochondria, but at a rate too slow to be useful in the study of labile phosphates.

Final experimental technique. A pair of centrifuge tubes were required for a complete analysis of a mitochondrial suspension. In most cases duplicate pairs were actually prepared. In one tube of each pair was placed 2·5 ml. of silicone fluid of the appropriate density (1·05 when 0·25 M-sucrose was used as a suspension medium). In the other tube of each pair was placed 1 ml. of formalin–$HClO_4$ fixative (sp.gr. 1·10), the tube weighed and 1·5 ml. of silicone fluid added. All tubes were then placed in an ice bath and 2 ml. of mitochondrial suspension at 0° (containing 30–50 mg. of dry mitochondria) was layered over the silicone in each tube. The tubes were then placed in the chilled high-speed head and the refrigerated centrifuge was accelerated by turning the handle to maximum as rapidly as possible. After 2·5 min. the centrifuge was switched off and allowed to stop. The tubes were removed one at a time from the centrifuge head and into each was placed a U-shaped piece of plastic-coated bell-wire (Fig. 1). The ends of the wire were bent at right angles at such a level that when inserted in the tube the ends rested on the lip of the tube and the bottom of the U was 3–5 mm. within the silicone layer. The tubes were placed in a mixture of ethanol and solid CO_2 and left until it was convenient to continue the manipulations.

One of the tubes was removed from the bath and allowed to warm until the solid supernatant could be lifted from the tube by traction on the ends of the

wire. The surface of the solid supernatant was wiped free from the adherent silicone with filter paper. The supernatant was suspended by means of the wire on the rim of a weighed 15 ml. conical centrifuge tube containing 1 ml. of 30 % (w/v) TCA and, on thawing, it dripped into the TCA and was deproteinized. On reaching room temperature the tubes were weighed and the contents mixed. After centrifuging to remove precipitated protein the solutions were decanted and stored at 2°.

As soon as the supernatant had been removed from the tube the liquid silicone was poured off, leaving either the $HClO_4$ extract or mitochondrial pellet still frozen. The bottom of the tube was pushed through a hole in a rubber stopper into a container of solid CO_2 (Fig. 2) and the whole inverted and allowed to drain. After about 30 sec. the inside rim of the tube was wiped with filter paper, and the tube removed from the container, filled with hexane at room temperature and returned to the ethanol–CO_2 bath. When thoroughly chilled the hexane was decanted and the tube allowed to drain while in the solid-CO_2 container. Two further hexane washings were necessary to remove all but traces of silicone from the tube.

After the last hexane washing the tube containing the formalin–$HClO_4$ extract was stoppered and allowed to warm to room temperature. The mitochondrial pellet in the tube without fixative usually became loose after the first hexane washing and was allowed to drop on filter paper, where excess of hexane rapidly evaporated without melting of the pellet. At this point the pellet was picked up with clean forceps and returned to its centrifuge tube, which was filled with hexane. After the third hexane washing the dried frozen pellet was put into a similar weighed centrifuge tube containing an accurately weighed portion of 10 % (w/v) TCA (1 ml.), and the tube stoppered. On occasions when the total dry wt. and the water content of the pellet were to be determined, the pellet was placed in a clean, weighed tube. When the tubes had attained room temperature the stoppers were removed and the remaining traces of hexane were allowed to evaporate. The tubes were then weighed after removal of moisture from the outside.

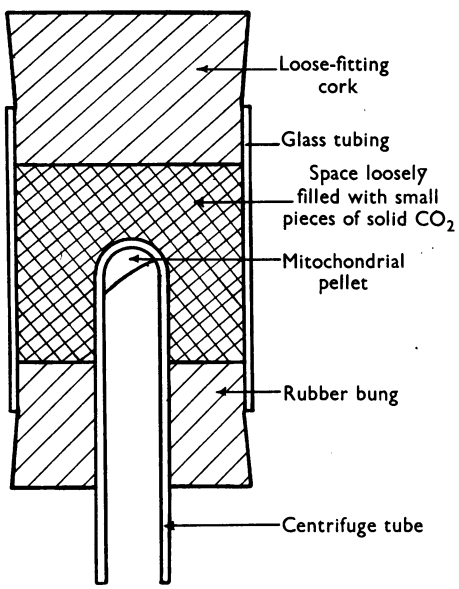

Fig. 2. Apparatus for draining silicone and hexane from frozen mitochondrial pellets.

The mitochondrial pellets in 10 % (w/v) TCA were broken into small fragments with a glass rod. The tubes containing TCA and formalin–$HClO_4$ were stored overnight at 2° and those containing the unextracted pellets were dried overnight in an oven at 105°.

The next morning the contents of the tubes containing the extracted pellets were stirred and the tubes centrifuged at 0° for 5 min. The extracts were decanted and stored at 2° to await analysis. The residues were reserved for determination of mitochondrial dry wt. as described in the next paragraph.

The tubes containing the dried pellets were cooled and weighed and the percentage dry wt. of the pellet was calculated.

Determination of 'acid-insoluble mitochondrial dry matter'. The TCA-extracted residues, after decantation of the extracting fluid, were washed once with 2 ml. of 5 % (w/v) TCA and once with 2 ml. of water. The residues were separated from the washing fluid each time by centrifuging for 5 min. at the full speed of the high-speed head of the centrifuge. Water (1 ml.) was added to the residue and the tubes were dried in an oven at 105° overnight. During this time remaining traces of TCA decomposed. The tubes containing the residues were then cooled and weighed. If to the weights of the acid-insoluble mitochondrial dry matter (subsequently referred to as M) so obtained was added the weight of sucrose and other solutes contained in the TCA extract, the sum agreed to within less than 1 % with that obtained by directly drying a similar mitochondrial pellet.

In some of the early experiments, the TCA washing of the residue was followed by two washings with 2 ml. of ethanol before drying in the oven. It was later discovered that these brief washings with ethanol removed about 10 % of the acid-insoluble solids. These results have therefore been corrected for this error. Since this correction is an approximation, the resulting values for M may have errors of as much as 5 %.

Dry weight of sedimented mitochondrial pellet. For purposes of comparison with mitochondria sedimented through silicone, a series of determinations were made on the dry wt. and water content of pellets centrifuged directly from mitochondrial suspensions at 0° without the use of silicone. The tubes were removed from the centrifuge and the supernatant was decanted. Then, with the tube maintained in an inverted position the internal walls of the tube were carefully dried with filter paper. Great care was taken to avoid touching the pellet. The tube containing the pellet was weighed, dried overnight at 105°, cooled and reweighed. These two weighings supplied the data for the calculation of the percentage dry wt. of the pellet.

Estimation of extra-particulate fluid. The accurate analysis of internal solute concentrations in mitochondria requires the estimation of the amount and solute content of the extra-particulate fluid of the pellet. The amount of this adherent medium may be determined from the distribution between the pellet and the medium of a high-molecular-weight substance, which neither penetrates the mitochondria nor is adsorbed on their surface. Polyglucose (Pacsu & Mora, 1950; Mora & Pacsu, 1955), of a molecular weight of 50000, is uncharged, spherical, very soluble in water, but only slightly hydrated. These properties suggest that it would be at least as suitable as inulin for the estimation of extra-particulate fluid.

Accordingly the distribution of polyglucose, inulin and sucrose in mitochondrial pellets sedimented from different media was investigated. For ease of analysis in the presence of sucrose the polyglucose and inulin were tagged with a terminal [^{14}C]carboxyl group. Sufficient labelled polyglucose was found to be present in the mitochondrial pellet sedimented from a suspension of mitochondria in sucrose to account for 19–22% of the total water of the pellet (Table 3). Since it seemed unlikely that the extra-particulate space could be much smaller than this, and that the use of polyglucose could lead to an underestimation, we have assumed that the distribution of labelled polyglucose in fact measures the extra-particulate fluid of the pellet and have included it in the medium in all further experiments in which this fluid was to be measured. Inulin has been extensively used as a measure of extracellular space in whole animals and tissue slices. Since inulin is not easily estimated in the presence of

Table 3. *Distribution of carboxypolyglucose, carboxyinulin and sucrose in mitochondrial pellets*

Volumes (2 ml.) of the indicated mitochondrial suspensions at 0° were centrifuged through silicone, the pellets washed with hexane and extracted with 10% (w/v) TCA. The supernatants and extracts were assayed for the three compounds.

Medium used for preparation and suspension of mitochondria	Expt. no.	Total water (%) 'penetrated' by: Carboxy-polyglucose	Carboxy-inulin	Sucrose	Internal water 'penetrated' by sucrose (%)
Sucrose	1	19·4	—	67	58
		20·1	—	66	57
		19·3	—	66	58
	2	22·2	—	68	58
		20·6	—	67	57
		20·5	—	69	59
Sucrose–EDTA	3	27·0	—	70	58
		26·5	—	69	57
	4	19·5	—	64	55
		18·1	—	63	55
Sucrose–EDTA + salt*	3	36·2	—	77	64
		33·2	—	76	64
		—	65	—	—
		—	68	—	—
Sucrose–EDTA + salt*	4	27·3	—	73	63
		25·3	—	70	60
Sucrose–PVP	5	44·7	—	77	56
		42·8	—	78	62
Sucrose–PVP + salt*	5	41·4	—	76	60
		40·4	—	77	63
Dialysed sucrose–PVP	6	30·8	—	82	74
		30·6	—	81	72
Dialysed sucrose–PVP + salt*	6	33·8	—	75	63
		31·4	—	76	64

* Salt indicates that the medium contained 0·04 molal KCl and 0·04 molal NaCl.

sucrose the distribution of [^{14}C]carboxyinulin was measured in a mitochondrial pellet sedimented from sucrose–EDTA solution. The results (Table 3) suggest that inulin had penetrated about two-thirds of the water of the pellet, although labelled polyglucose in a similar preparation had penetrated only one-third of this water. Thus the mitochondria appear to be appreciably permeable to carboxyinulin. Whether this is due to true penetration or merely to adsorption is not clear.

Permeability of mitochondria to sucrose. Although sucrose has been used by most workers in fractionation of cell particulates, its function remains obscure. It has generally been assumed that it does not penetrate the mitochondria, as measurements of the volume changes of mitochondria in sucrose solutions of varying strength indicate that they act as osmometers (for example, see Tedeschi & Harris, 1955). The data for sucrose in Table 3 indicate that this solute penetrates a large proportion of the intramitochondrial water. The amount of intramitochondrial sucrose is sufficient to justify the assumption that 57–59 % of the intramitochondrial water had the same sucrose concentration as the external medium. In other words, the 'sucrose space' within the mitochondria was about 60 % of the total water space.

Table 3 also shows that the internal sucrose space of sucrose–EDTA mitochondria was the same as that of sucrose mitochondria in spite of varied polyglucose spaces. The sucrose space of the mitochondria was appreciably increased by the addition of NaCl and KCl at final concentrations in the medium of 0·04 molal (Table 3, Expts. 3 and 4).

In Table 3 are also shown the polyglucose and sucrose 'spaces' of mitochondria isolated from PVP media. In Expt. 5 it was observed that the sucrose space, both in the presence and absence of KCl (0·04 molal) and NaCl (0·04 molal), was similar to that in sucrose–EDTA plus the same salt mixture. In Expt. 6, the use of dialysed PVP resulted in a greatly increased sucrose space in the salt-free medium. This space was reduced on the addition of NaCl and KCl to the medium, suggesting that the salt content of the undialysed PVP was responsible for the somewhat lower values in Expt. 5 before the medium had been supplemented with the salt mixture.

Calculation of internal solute concentration of mitochondria. The weight of the mitochondrial pellet may be considered to be composed of four separate weights: that of the extracted, washed and dried mitochondrial residue (M), that of its soluble internal solutes (S), that of its internal water (I) and that of the adherent supernatant fluid (E).

Calculation of the amounts of acid-soluble solutes in the mitochondrial pellet ($S+E$) requires the estimation of the volume of the acid extract of the pellet. This was taken to be the volume of the TCA (1 ml.) + the wt. of the mitochondrial pellet ($\sim$0·15 g.) $-M$ ($\sim$0·04 g.). No correction has been made for the density of the fluid content of the pellet, which must be similar to that of the suspending medium (1·035–1·050). The error due to this approximation is small ($\sim$0·5 %).

The volume of E was calculated from the measurements of radioactivity in the mitochondrial extract and the supernatant fluid. The measurement of the density of the medium allowed calculation of the weight of E.

The total content of the various solutes of the mitochondrial extract was calculated from the results of the chemical determinations. The concentrations of the same solutes in the supernatant fluid were also determined, and thus the contribution of adherent medium to the total solute content of the pellet was calculated from the known volume E. Subtraction of these amounts yielded the amounts of the internal mitochondrial solutes (S). (In this calculation the molecular weight of the organic phosphates was assumed to be approx. 300.) As not all soluble components of the mitochondria were measured, S must be low; however, by independent measurement it was found that this deficit amounted to no more than 0·3 mg./100 mg. wet wt. of mitochondria. This slight deficit was ignored. It should be noted that S is only about 3 % of the total weight of the mitochondrial pellet and largely comprises sucrose.

From the total weight of the mitochondrial pellet and the values of M, S and E, the weight (and volume) of the internal water (I) was calculated by difference.

The concentrations of the individual internal solutes making up S were obtained by dividing by I. These concentrations are thus expressed on a millimolal basis.

It should be noted that the mitochondria spun into the formalin–$HClO_4$ fixative were used only for the determination of labile compounds (phosphates), as the remainder of the solutes could only be reliably estimated on the TCA extracts.

The indirect calculation of I described above has been found to agree to within 1 % with that obtained by measuring the water content of $I+E$ (loss in weight on drying of the mitochondrial pellet) and deducting the contribution of E.

Water content of mitochondria. To our knowledge there are no published values for the water content of mitochondria prepared and maintained in 0·25 M-sucrose at 0°. Knowledge of this value would appear essential for evaluation of the degree of swelling occurring when these mitochondria are suspended in other media or at elevated temperatures.

Therefore measurements were made on mitochondria at 0° prepared in 0·25 M-sucrose and resuspended in 0·25 M-sucrose with or without the additions given in Table 4. The percentage dry weights of sedimented mitochondrial pellets were determined by drying at 105°. Table 4 shows that whether or not the mitochondria passed through silicone the percentage dry wt. of the pellet was the same. All the preparations studied yielded a pellet with a percentage dry wt. greater than 32. The only published values for mitochondrial dry wt. (MacFarlane & Spencer, 1953; Price & Davies, 1954; Fonnesu & Davies, 1955; Klemperer, 1955) are of the order of 20 %. The mitochondria studied by these workers were under their conditions already swollen to twice the water content that they presumably had when they were at 0° in sucrose alone.

The addition of 0·1 vol. of a 9 % (w/v) labelled polyglucose solution to a suspension of mitochondria in 0·25 M-sucrose, with or without the addition of NaCl and KCl to give final concentrations of about 0·04 molal, caused a slight extrusion of water from the particles (5–10 %). This is probably due to the colloid osmotic pressure exerted by the polyglucose and is additional evidence that this substance does not penetrate the mitochondria.

It is apparent that the dry wt. of the pellet as presented above will vary with the amount of solutes dissolved in the intramitochondrial and the extraparticulate fluids of the pellet. Thus changes in percentage dry wt. (as defined above) incident on changes in medium or conditions may give erroneous impressions of shrinking or swelling. The measurement of the interstitial fluid by the use of labelled polyglucose enables the internal mitochondrial water (I) and the non-extractable solids (M) to be calculated. The ratio of these two values, I/M, is a more sensitive and meaningful estimate of mitochondrial hydration permitting comparison of mitochondria in widely differing suspension media.

Solute content of mitochondria prepared in 0·25 M-sucrose at 0°

While a fairly consistent pattern for the content of some solutes of mitochondria at 0° was observed, others were subject to considerable variation. Table 5 gives the values observed in three representative experiments. The three most consistent of the components studied were potassium, orthophosphate and organic phosphate. Chloride was subject to great variation and may be present in the mitochondria only as a contaminant. Although the external concentrations of sodium and potassium were similar (about 0·7 millimolal), the particles retained about 10 times more potassium than sodium (50 millimolal K, 6 millimolal Na). A large and consistent proportion of internal phosphate

Table 4. *Percentage dry weight of centrifuged mitochondrial pellets*

Volumes (2 ml.) of mitochondrial suspensions at 0° in 0·25 M-sucrose containing the indicated additions were centrifuged for 5 min. at 25 000 *g*. Some of the suspensions were layered over 2·5 ml. of silicone. The concentration of labelled polyglucose was 0·9 %, of KCl 0·04 M, of NaCl 0·04 M. When silicone was present the tubes were washed with hexane by the procedure described in the text. When silicone was absent the supernatant was decanted and the tube wiped with filter paper. After the residues had been weighed they were dried overnight at 105° and reweighed. The values in the table are weight of dry residue × 100/wt. of wet residue.

Additions			No silicone			Silicone		
Labelled polyglucose	KCl	NaCl	% dry wt.	S.E.M.	No. of expts.	% dry wt.	S.E.M.	No. of expts.
−	−	−	33·9	0·3	(10)	.	.	.
+	−	−	35·3	0·3	(4)	.	.	.
−	+	−	32·5	0·2	(18)	32·7	0·3	(14)
−	+	+	33·0	0·4	(8)	32·3	0·7	(8)
+	+	+	.	.	.	34·5	0·2	(16)

Table 5. *Concentrations at 0° of internal solutes of mitochondria prepared in sucrose solutions*

Samples (2 ml.) of mitochondrial suspensions at 0° were sedimented through silicone as described in the text. All preparations contained labelled polyglucose. I, Weight of internal water; M, weight of dry matter in mitochondria.

Expt. no.	Millimolal K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	47	5	32	6·8	36	84	—
2	48	4	17	8·2	44	84	1·91
3	59	9	1	7·5	39	84	1·63
External concn.	0·8	0·6	2·3	0·10	0·26	—	—

(84 %) was in the form of organic esters, roughly half of which were adenine nucleotides. The total concentration of the phosphate esters was somewhat less than that of the potassium.

Solute content of mitochondria prepared in other media at 0°

Sucrose–EDTA. EDTA, according to Beaufay, Hers, Berthet & Duve (1954) and Slater & Cleland (1953), appears to stabilize certain mitochondrial enzymes. It has been widely used and is generally assumed to have no undesirable effects. The solute composition of mitochondria prepared in and separated from 0·25 M-sucrose containing 0·001 M-EDTA at 0° is shown in Table 6. As in sucrose mitochondria, the chloride concentration showed considerable variation and in this case potassium, although lower, was also somewhat variable. The organic phosphate concentration was somewhat lower (31 millimolal) but the percentage organic phosphate was much higher (92 %). Thus the most striking difference from sucrose mitochondria was the low orthophosphate concentration. The water content (as indicated by the values of I/M) appeared somewhat higher.

Sucrose–PVP. Novikoff (1956) has described the use of PVP as an addition to sucrose in the preparation of mitochondria. He states that mitochondria prepared in this medium retain the thread-like shape characteristic of their intracellular condition. Electron-microscope studies have shown that the mitochondria in the sucrose–PVP medium retain the *cristae mitochondriales* intact. Dalton (1957) showed that in mitochondria prepared in 0·25 M-sucrose the *cristae* were frequently ruptured; as might be expected in view of this finding, mitochondria isolated from 0·25 M-sucrose are not rod-like but tend towards a spherical shape. In contrast, mitochondria from tissues homogenized in 0·25 M-sucrose containing 7·3 % of PVP appeared rod-like when observed with phase-contrast microscopy. The solute concentrations of mitochondria prepared in PVP media at 0° are given in Table 7. In the third experiment the PVP was exhaustively dialysed against distilled water before use. The retention of potassium was much less by the PVP mitochondria than by the sucrose mitochondria (average about 20 millimolal in PVP against about 50 millimolal in sucrose). The potassium retention was even less when the PVP had been dialysed. As was previously observed for the EDTA mitochondria the orthophosphate concentration was only about one-third of that found in the sucrose mitochondria. The ratio of organic phosphate to total phosphate did not show the same constancy exhibited by the sucrose mitochondria.

The water content as indicated by the values of $I:M$ give no support to the concept that the PVP mitochondria are less swollen than sucrose mitochondria. It would appear that rupture of the *cristae* involves a change in shape without a marked change in hydration.

Dextran mitochondria. The possibility that PVP exerted its action by virtue of its colloidal nature suggested that other similar substances might be used for keeping mitochondria in the rod-shaped state. Dextran is one such substance, and Hogeboom (1955) has stated that liver mitochondria

Table 6. *Concentrations at 0° of internal solutes of mitochondria prepared in sucrose–EDTA solutions*

Details are as in Table 5.

	Millimolal						
Expt. no.	K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	27	4	21	1·9	32	94·5	2·18
2	36	8	39	3·2	30	90	1·96
External concn.	0·8	0·7	0·3	0·10	0·29	—	—

Table 7. *Concentrations at 0° of internal solutes of mitochondria prepared in sucrose–PVP solutions*

Details are as in Table 5. In Expt. 3 the PVP was exhaustively dialysed before use.

	Millimolal						
Expt. no.	K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	15	8	39	1·5	45	97	1·81
2	25	8	34	0·9	50	98	1·75
3	7	6	27	2·2	28	93	1·82
External concn.	2·0	0·8	0·3	0·27	0·83	—	—

prepared in isotonic sucrose containing 6–12 % of dextran retain their normal rod-like shape (see also Birbeck & Reid, 1956). Mitochondria isolated from 0·25 M-sucrose containing 6 % of dextran at 0° were not rod-like under our conditions. From the analysis of the external medium it appears that the undialysed dextran contained an appreciable amount of NaCl. The potassium retention in dextran medium was very low (Table 8). Orthophosphate concentration was also lower than in sucrose mitochondria, but more striking was the low concentration of the organic phosphates (about 23 millimolal). It is uncertain whether the swollen state of the mitochondria in the second experiment (I/M 3·66) was related to the fact that the dextran was undialysed.

'Supernatant' mitochondria. The cell sap may contain cofactors which play a part in preserving the morphological and physiological integrity of the mitochondria in the intact cell. The loss of these substances on washing with sucrose solutions may be the cause of the appearance of the characteristic 'fluffy' layer and perhaps cause other changes in the mitochondria themselves. Table 9 contains data of the solute concentrations of mitochondria washed with and resuspended in 'sucrose supernatant' prepared as already described. The sodium, potassium and chloride values resemble those for PVP mitochondria much more than those for sucrose mitochondria, but the organic and orthophosphate values are similar to those for sucrose mitochondria. Although this preparation was grossly swollen, the value for organic phosphate was not reduced; thus the content of organic phosphate was 50 % greater than in any of the other preparations. Phosphate loss thus appears to be reduced in this medium.

Solute distribution in mitochondria at 0° in the presence of NaCl *and* KCl. In the previous section we have described the retention of simple ions by thoroughly washed mitochondria at 0°. These ions therefore are bound under this set of circumstances. On the addition of a mixture of NaCl and KCl, the concentrations of sodium, potassium and chloride are appreciably higher in the mitochondrial water than in the medium. The excess amount (m-moles/kg. of M) of such ions is roughly the same as was present in the mitochondria before the addition of the salt, indicating that these ions are still bound. Table 10 illustrates this finding with respect to potassium. Similar results were obtained for the distribution of added sodium and chloride. The addition usually caused a shrinkage (10–25 %) of the mitochondria. In three of the media shrinkage did not occur. In the dialysed PVP medium there was no change in hydration and with the dialysed dextran and 'supernatant' media swelling occurred on the addition of the salt mixture.

Effect on the internal phosphates of the addition of nucleotides at 0°. To suspensions of mitochondria at 0° to which salt (final molal concentration of NaCl and KCl was 0·04) had been added was subsequently added a solution of a commercial preparation of adenosine triphosphate (ATP) to raise the total external phosphate concentration to 0·01–0·015 molal. This nucleotide solution contained (on a phosphate basis) 44 % of ATP, 31 % of adenosine diphosphate, 7 % of adenosine monophosphate, 6 % of orthophosphate and 12 % of unidentified phosphates. As shown in Table 11, the addition of

Table 8. *Concentrations at 0° of internal solutes of mitochondria prepared in sucrose–dextran solutions*

Details are as in Table 5.

Expt. no.	Type of dextran used	Millimolal K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	Benger's (dialysed)	7	5	46	2·7	23	90	1·85
External concn.		1·0	0·6	0·8	0·2	0·6	—	—
2	Glaxo (undialysed)	9	14	31	4·7	20	82	3·66
External concn.		0·6	4·7	3·1	0·12	0·35	—	—

Table 9. *Concentrations at 0° of internal solutes of mitochondria prepared in 'supernatant'*

Details are as in Table 5.

Expt. no.	Millimolal K	Na	Cl	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
1	21	9	35	6·3	48	88	2·45
External concn.	13	5	5	1·6	4	—	—

this nucleotide solution caused a large increase in orthophosphate concentration of sucrose mitochondria. Concomitantly the percentage of organic phosphate decreased, although there was no decrease in organic phosphate and the water content showed only slight changes. In contrast the addition of the nucleotide mixture to sucrose–PVP mitochondria in the salt-containing medium caused only a slight increase in intramitochondrial orthophosphate concentration, and thus had no effect on the proportion of organic phosphate, but caused the mitochondria to swell by about 20%.

Effect of 2:4-dinitrophenol (DNP) on mitochondrial solutes at 0°. As DNP has been used in many investigations on tissue preparations it was of interest to investigate its effect on the internal solutes of mitochondria. Table 12 shows the effect of the addition of DNP (2×10^{-4} M) to a suspension of mitochondria at 0° in 0·25 M-sucrose and in 0·25 M-sucrose containing 0·04 molal KCl and 0·04 molal NaCl. In both cases this addition resulted in a marked loss of 'bound' potassium and a concomitant loss in phosphates. The phosphate loss from mitochondria isolated from the sucrose medium was equally contributed to by organic and orthophosphate. The phosphate loss from mitochondria isolated from the salt-containing medium was apparently confined to orthophosphate.

The effect of DNP on mitochondrial hydration appears to vary according to the experimental conditions (MacFarlane & Spencer, 1953; Price & Davies, 1954; Klemperer, 1955). Under our conditions, at 0°, DNP added to a mitochondrial suspension in 0·25 M-sucrose caused the same degree of shrinkage as that which occurred on the addition of the salt mixture to the same suspension (cf. line 1

Table 10. *Effect of salt on the binding of potassium by mitochondria at 0°*

Details are as in Table 5. Salt, when added, raised the external concentration to about 0·04 molal KCl and 0·04 molal NaCl. Excess concentrations represent the numerical difference in concentration between mitochondria and medium. M, mitochondrial dry wt.

	Excess concn. (millimolal)		Excess content (m-moles/kg. of M)		Relative water content (I/M)	
Mitochondrial suspension medium	Without salt	With salt	Without salt	With salt	Without salt	With salt
Sucrose	46	42	—	—	—	—
Sucrose	47	57	90	97	1·91	1·71
Sucrose	58	53	95	77	1·63	1·45
Sucrose	—	45	—	77	—	1·70
Sucrose–EDTA	26	29	57	46	2·18	1·60
Sucrose–EDTA	35	44	87	53	1·96	1·50
Sucrose–PVP	13	28	23	45	1·81	1·62
Sucrose–PVP	23	29	40	43	1·75	1·47
Sucrose–dialysed PVP	5	0	9	0	1·82	1·82
Sucrose–dextran (Benger's dialysed)	6	1	11	2	1·85	2·14
Sucrose–dextran (Glaxo undialysed)	8	8	29	24	3·66	2·96
'Supernatant'	8	10	20	28	2·45	2·79

Table 11. *Effect of 'ATP' on the internal phosphate of mitochondria at 0°*

Details are as in Table 5. All suspensions contained approx. 0·04 molal KCl and 0·04 molal NaCl. The 'ATP' preparation was that described in the text. M, mitochondrial dry wt.

			Millimolal		m-moles/kg. of M			
Suspension medium	Additions	Fluid analysed	Ortho-phosphate	Organic phosphate	Ortho-phosphate	Organic phosphate	Organic phosphate (%)	Relative water content (I/M)
Sucrose	None	Mitochondrial	6·1	41	10·2	68	87	1·67
		Medium	0·06	0·2	—	—	—	—
	'ATP'	Mitochondrial	17	42	28	69	72	1·65
		Medium	1·2	10·9	—	—	—	—
Sucrose	None	Mitochondrial	8·0	44	13·6	75	85	1·70
		Medium	0·05	0·2	—	—	—	—
	'ATP'	Mitochondrial	20	50	35	88	72	1·75
		Medium	1·5	11·7	—	—	—	—
Sucrose–PVP	None	Mitochondrial	3·0	41	4·9	67	93	1·62
		Medium	0·01	1·0	—	—	—	—
	'ATP'	Mitochondrial	3·9	44	7·5	85	92	1·93
		Medium	0·3	17·8	—	—	—	—

Table 12. *Effect of 2:4-dinitrophenol on the internal potassium and phosphates of mitochondria at 0°*

Details are as in Table 5. Mitochondria were prepared and suspended in 0·25 M-sucrose. M, mitochondrial dry wt.

Additions	Mitochondrial content (m-moles/kg. of M)			External concn. (millimolal)		Relative water content (I/M)
	Excess K*	Content of: Ortho-phosphate	Content of: Organic phosphate	Ortho-phosphate	Organic phosphate	
None	95	12·2	64	0·09	0·27	1·63
2×10^{-4} M-DNP	59	8·0	59	0·28	0·33	1·40
0·04 M-KCl + 0·04 M-NaCl	77	10·4	61	0·08	0·23	1·45
0·04 M-KCl + 0·04 M-NaCl + 2×10^{-4} M-DNP	56	3·2	61	0·32	0·25	1·69

* Calculation as in Table 10.

with lines 2 and 3, Table 12). The addition of DNP to a mitochondrial suspension containing the salt mixture, on the other hand, caused a swelling of the same order as the previous shrinkage.

DISCUSSION

Knowledge of the solute contents of mitochondria at 0° before possible degenerative changes is essential to an understanding of the effects of various experimental conditions at elevated temperatures. It is worth emphasizing that all the studies reported here were conducted at 0°.

Throughout this work the assumption has been made that the internal mitochondrial solution represents a single aqueous phase. Our calculations are thus based on the even distribution of the internal solutes within the internal water. The distribution of added potassium, sodium and chloride appears to be in agreement with this assumption, but the partition of sucrose suggests that the whole of the mitochondrial water may not be available to this substance. The fact that the internal sucrose concentration is considerably less than the external would, on the basis of a single aqueous phase, imply a mechanism for the partial exclusion of sucrose. However, an inert uncharged molecule such as sucrose would be expected to be passively distributed, and an alternative explanation for these results would be that there are two internal aqueous phases, the concentration of sucrose in the more accessible of these compartments being the same as that in the external medium. The apparent partial penetration of mitochondria by sucrose could also be explained on the basis of varying permeability of the mitochondrial population. Thus two sorts of mitochondria might be visualized, one completely permeable to sucrose and the other impermeable. It is impossible as yet to distinguish between the various possibilities. The observation that the sucrose space of mitochondria prepared in dialysed sucrose–PVP medium was reduced on the addition of a mixture of sodium, chloride and potassium chloride seems to favour the hypothesis that part of the mitochondrial water is impermeable to sucrose.

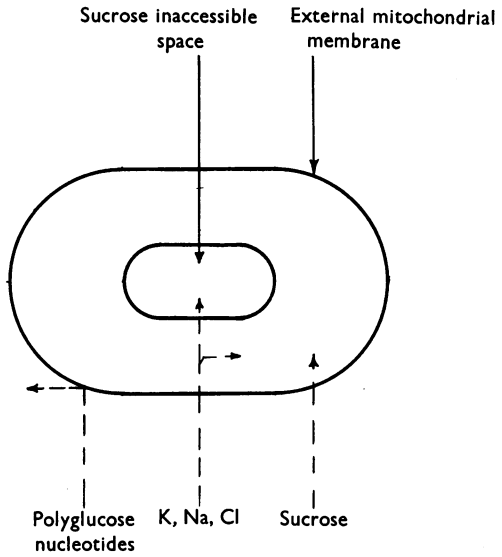

Fig. 3. Diagrammatic representation of the permeability of mitochondria to various solutes. The broken lines show areas of the mitochondria accessible to the solutes.

Our experiments show that the addition of a nucleotide mixture to a mitochondrial suspension produced no change in the internal organic phosphate concentration. This may be due to the impermeability of the mitochondrial membrane to these nucleotides. Fig. 3 summarizes these observations in diagrammatic fashion.

Our results indicate that quantitatively the chief intraparticulate ions retained in washed mitochondria are potassium and phosphates. Thus mitochondria appear to exhibit the same selectivity for potassium and phosphates as intact cells when incubated in a saline medium. However, the quantities retained by these washed mitochondria are small compared with the total intracellular

content, and it is therefore impossible at present to conclude that the mitochondria are responsible for the ionic selectivity of whole cells.

SUMMARY

1. Rapid isolation of rat-liver mitochondria from their suspending medium has been achieved by centrifuging them into a layer of silicone fluid. A high-density aqueous 'fixative' beneath the silicone stabilized labile phosphate esters when these were to be measured. In the absence of this fixative 19% of the mitochondrial acid-soluble organic phosphate was hydrolysed in 5 min. at room temperature.

2. In this procedure the centrifuge tubes were cooled in a solid carbon dioxide–ethanol bath, the frozen aqueous supernatant fluid was removed, the silicone poured off, and residual silicone removed from the lower layer by washing with hexane at low temperature.

3. The volume of extra-particulate fluid in the mitochondrial pellets as estimated from the distribution of radioactive polyglucose between pellet and medium represented 19–22% of the total water of mitochondrial pellets isolated from 0·25M-sucrose solutions at 0°.

4. Mitochondria were found to be permeable to sucrose. The internal concentration of sucrose in mitochondria isolated from 0·25M-sucrose solutions at 0° was about 60% of that in the medium. This was tentatively taken to mean that only 60% of the mitochondrial water was permeable to sucrose.

5. The percentage dry wt. of mitochondrial pellets isolated from 0·25M-sucrose at 0° was 33·9. The presence of polyglucose in the external medium increased this value consistently to 35·3.

6. The concentrations (millimolal) of potassium, orthophosphate and organic phosphate in the internal water of mitochondria isolated from 0·25M-sucrose at 0° were about 50, 7 and 40. The use of other salt-poor isolation and suspending media did not result in higher values for these components.

7. Increase in the concentration of sodium chloride and potassium chloride in the various suspending media in each case by about 0·04 molal caused roughly the same increase in the concentration of these salts in the mitochondrial water. Thus these added substances appear to be passively distributed between mitochondria and medium, and the 'bound' ions, in particular potassium, remain fixed within the mitochondria.

8. The addition of adenine nucleotides and of 2:4-dinitrophenol to the suspending medium at 0° caused changes in the mitochondrial phosphates and potassium.

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