Biochem. J. (1972) **126**, 689–700
Printed in Great Britain

The Effect of the Coupled Oxidation of Substrate on the Permeability of Blowfly Flight-Muscle Mitochondria to Potassium and other Cations

By RICHARD G. HANSFORD* and ALBERT L. LEHNINGER
Department of Physiological Chemistry, The Johns Hopkins University, School of Medicine, Baltimore, Md. 21205, U.S.A.

(*Received* 8 *September* 1971)

1. Blowfly flight-muscle mitochondria respiring in the absence of phosphate acceptor (i.e. in state 4) take up greater amounts of K^+, Na^+, choline, phosphate and Cl^- (but less NH_4^+) than non-respiring control mitochondria. 2. Uptake of cations is accompanied by an increase in the volume of the mitochondrial matrix, determined with the use of [^{14}C]-sucrose and ^3H_2O. The osmolarity of the salt solution taken up was approximately that of the suspending medium. 3. The [^{14}C]sucrose-inaccessible space decreased with increasing osmolarity of potassium chloride in the suspending medium, confirming that the blowfly mitochondrion behaves as an osmometer. 4. Light-scattering studies showed that both respiratory substrate and a permeant anion such as phosphate or acetate are required for rapid and massive entry of K^+, which occurs in an electrophoretic process rather than in exchange for H^+. The increase in permeability to K^+ and other cations is probably the result of a large increase in the exposed area of inner membrane surface in these mitochondria, with no intrinsic increase in the permeability per unit area. 5. No increase in permeability to K^+ and other cations occurs during phosphorylation of ADP in state 3 respiration.

For some time there has been general consensus that the inner mitochondrial membrane is essentially impermeable to alkali-metal cations. This view, originally suggested by the observation that a portion of the mitochondrial K^+ is retained during sucrose washing (Spector, 1953; Bartley & Davis, 1954; Amoore, 1960) and by the osmotic behaviour of mitochondria in solutions of potassium salts (Chappell, 1954; Tedeschi, 1961), was strengthened by the demonstration of a low rate of exchange of endogenous K^+ with added ^{42}K (Gamble, 1957; Amoore & Bartley, 1958). Such exchange experiments removed the ambiguity of earlier work in which gross fluxes of K^+ were involved, in that the latter are always associated with the movement of at least one other ion, which may be the limiting factor. More recently many studies have been reported on the induction of a permeability to alkali-metal cations by certain ionophorous antibiotics (Moore & Pressman, 1964; Pressman, 1965; Cockrell *et al.*, 1966; Harris *et al.*, 1966; Azzi & Azzone, 1966); this work has also emphasized the view that the mitochondrial membrane is intrinsically impermeable to K^+. However, Caswell (1969) has reported a transient increase in permeability induced by uncoupling agents or a combination of uncoupling agents and respiratory inhibitors, and Mitchell & Moyle (1967, 1969) have suggested that the liver mitochondrion is permeable to Na^+ and slightly to K^+ in the sense that these cations enter in exchange (antiport according to the terminology of Mitchell, 1967) for H^+. In addition, two groups of workers have shown a greatly increased permeation of ox heart mitochondria suspended in iso-osmotic potassium acetate solutions on the addition of a respiratory substrate (Brierley *et al.*, 1968; Blondin & Green, 1969). The increased permeation was attributed to an increased permeability, but this was not an unequivocal conclusion, as the effect of a superimposed membrane potential has also to be considered.

In the present paper we describe the interaction of blowfly muscle mitochondria with K^+ and other cations. Our observations indicate that state 4 respiration (see Chance & Williams, 1955) induces a large increase in permeability to K^+ and also to larger cations such as choline$^+$ and ornithine$^+$. The permeation of cations under these conditions requires a potential gradient and is thus an electrophoretic process; it differs from the neutral antiport of K^+/H^+ and Na^+/H^+ described by Mitchell & Moyle (1967, 1969).

Materials and Methods

Preparation of mitochondria

Flight muscles were expressed from the thoraces of 50–100 blowflies and gently homogenized in a Dounce homogenizer with a Teflon pestle in a volume of 40ml

* Present address: Department of Biochemistry, University College, Cardiff CF1 1XL, U.K.

of ice-cold preparation medium. This comprised 0.25M-sucrose, 5mM-tris–HCl and 1mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate], adjusted to pH7.7 at 0°C. The proteolytic enzyme Nagarse (obtained from Serva Feinbiochemica G.m.b.H., Heidelberg, Germany) was added, 0.5mg being used per ten flight muscles. This ratio and the pH are critical factors in the success of this preparation. Digestion was allowed to proceed for 10min at 0°C; during this time intact muscle bundles were dispersed with further gentle homogenization. The suspension was then filtered through four thicknesses of washed cheese-cloth and centrifuged at 10000*g* for 2min in a Sorvall RC2B refrigerated centrifuge. The resulting pellets were gently resuspended by using an ice-filled test-tube, made up to 40ml, and centrifuged again in the same fashion. As a routine the final pellet was suspended in 2ml of preparation medium. This method of preparation yielded mitochondria exhibiting respiratory-control ratios of more than 20 with pyruvate as substrate. In addition, the electron microscope revealed homogeneous populations of mitochondria, containing essentially no components of non-mitochondrial origin. If digestion was excessive, grossly distorted mitochondria were observed.

Incubation studies

Mitochondria were sedimented by centrifugation in a Sorvall RC2B centrifuge, in 10ml polypropylene tubes. The procedure was to allow the centrifuge to accelerate maximally to 19000 rev./min and then to brake maximally. This yielded tightly packed pellets from which supernatant fluid could be easily drained. The sides of the tubes were then very carefully wiped with a tissue and the pellets resuspended in 0.1ml of water or 0.25M-sucrose, followed by addition of 0.5ml of 10% (w/v) trichloroacetic acid. Precipitated protein was sedimented by centrifugation and the trichloroacetic acid extract was used for the determination of ions and the counting of radioisotopes.

The general plan was to compare energized (i.e. respiring and coupled) mitochondria with identical non-energized control systems containing uncoupling agent. Several authors have criticized the use of centrifugation to terminate incubations of this sort, on the grounds that the mitochondria might become anaerobic during the separation procedure (see, for example, Harris *et al.*, 1966). Although the values reported here for the differences between energized and non-energized mitochondria are probably underestimated, there are several reasons for believing that anaerobiosis of the pellets is not an important factor. First, the results obtained were the same whether air-saturated or oxygen-saturated media were used, though the latter were normally used in practice to allow longer incubation periods. Clearly, the possibility of the mitochondria becoming anaerobic before forming a pellet would be greater in the air-saturated medium. Secondly, essentially the same results were obtained in studies with an MSE 18 centrifuge, which accelerates more slowly and thus increases the risk of anaerobiosis before formation of the pellet. Thirdly, results to be presented in this study suggest that the mitochondrial membrane is impermeable to cations in the absence of energy. Thus, even should anaerobiosis occur, cations previously accumulated would be trapped within the mitochondria. Moreover, blowfly mitochondria are well suited to this approach, since they sediment very rapidly, especially in salt media.

Counting of radioactivity of samples

A sample (0.2ml) of the trichloroacetic acid extract was counted for radioactivity in 15ml of Bray's (1960) fluid in a Packard Tri-Carb liquid-scintillation counter. For the dual- and triple-channel counting the principles described by Hunter & Brierley (1969) were adopted. Windows and gain were set so that there was no overlap of the ^3H counts into the ^{14}C channel, and no overlap of the ^{14}C counts into the ^{36}Cl channel. The estimation of spaces was based on the comparison of the counts in the pellet extract with the counts in a sample of supernatant medium from the same tube, diluted to give a similar count rate and an identical concentration of trichloroacetic acid.

K^+ and Na^+ were determined by flame photometry in an EEL flame photometer, by employing a lithium internal standard, in standard and unknown solutions. NH_4^+ was determined by the ninhydrin technique of Rosen (1957).

Phosphate was determined by the 2-methylpropan-2-ol–stannous chloride method of Berenblum & Chain (1938). Interference from endogenous ATP breakdown during the formation of the pellets or in subsequent extraction and assay was probably of minor importance since the total adenine nucleotide content of blowfly mitochondria is only 14nmol/mg of protein (Price & Lewis, 1959).

Swelling was followed by recording the decrease in E_{700} of a mitochondrial suspension with a Beckman–Gilford spectrophotometer. Protein was determined by the biuret method with crystalline bovine serum albumin as standard. Water distilled twice from glass was used throughout and reagents were of the highest purity available commercially. Blowflies were provided generously and continually by Dr. B. Sacktor.

Results

Uptake of cations and phosphate or acetate by respiring blowfly flight-muscle mitochondria

Paired experiments were carried out in which mitochondria were suspended in iso-osmotic salt

media containing 10–15mM-potassium phosphate, but no phosphate acceptor, in the presence or absence of an oxidizable substrate; they were then centrifuged down as described in the Materials and Methods section. Analysis of the pellets revealed a large increment in cation and in phosphate in the pellets formed from respiring mitochondria compared with the non-energized controls. A detailed analysis of one such experiment, involving a KCl medium, is presented in Table 1. In the approx. 60s of incubation there was a large gain in K^+ and phosphate, and a small gain in Cl^-. At the same time there was a large increase in the matrix volume, as measured by the [^{14}C]sucrose-inaccessible space, such that the concentration of electrolyte in the expanded matrix essentially equalled that in the suspending medium (267mOsM, i.e. 267 milliosmoles/l). The mean matrix values quoted were obtained by substracting, for each pellet, the quantity of each ion that would be expected in the sucrose-accessible space assuming that its concentration is the same as in the medium, and then taking the means of the quantities remaining. This procedure thus removes variations from one pellet to another caused by contamination with the supernatant medium. On the other hand, for uncoupled mitochondria, the subtraction involves a small difference between two large numbers and is therefore open to error. For this reason, the matrix osmolarity calculated for the coupled mitochondria (283mOsM) is the more reliable value.

For interpretation of calculations of this nature it must be pointed out that only the total ion content of the sucrose-inaccessible space is measured; although most of this is probably in the matrix space, some may be adsorbed on the membrane. This may be the reason for the excess of chloride space over sucrose space in this experiment, although there is some evidence for entry of Cl^- into these mitochondria when the membrane is stretched, and this will be presented below. In experiments of this sort involving five different mitochondrial preparations, the matrix volume of non-energized blowfly flight-muscle mitochondria was found to be $1.00 \pm 0.07\,\mu$l/mg of total mitochondrial protein.

The composition of the medium used in the experiment reported in Table 1 was essentially that used in most polarographic experiments with blowfly mitochondria. Since phosphate was the main counter-ion involved, experiments were carried out to see if the presence of phosphate was obligatory for a large uptake of K^+. It emerged that there was only slight accumulation of K^+ in the absence of phosphate, this being an excess of 36nequiv. of K^+/mg of protein over the content of the control (respiration-inhibited) incubation. In the presence of 4mM-potassium phosphate the accumulation rose to 185nequiv./mg, and in the presence of 10mM-phosphate to 282nequiv./mg. It is noteworthy that Cl^- will not support an

Table 1. *Measurement of K^+, Na^+, Cl^- and phosphate content and of sucrose-inaccessible water of respiring and non-respiring blowfly flight-muscle mitochondria*

The basic medium comprised 0.12M-KCl, 15mM-potassium phosphate, pH7.1, 5mM-sodium DL-glycerol phosphate, pH7.1, 1mg of bovine serum albumin/ml, $0.5\,\mu$Ci of ^3H_2O/ml, $0.3\,\mu$Ci of ^{36}Cl/ml and $0.3\,\mu$Ci of [^{14}C]sucrose/ml. This was saturated with oxygen and 4ml portions were used for each incubation. In those incubation mixtures described as 'energized', 0.25ml of mitochondrial suspension (22.2mg of protein/ml) was added to each tube. Tubes were centrifuged 40s after mixing, as described in the Materials and Methods section. In the 'non-energized' incubation mixtures, KCN and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were added to 2.5mM and $1\,\mu$M respectively before the addition of mitochondria. 'Energized' incubation mixtures were centrifuged individually but the four 'non-energized' incubation mixtures were centrifuged together. The temperature was 25°C. Extracts of the mitochondrial pellets were made and assayed as described in the Materials and Methods section. All values quoted are the means ±S.D. of the results from four incubations.

	Non-energized	Energized
Spaces (μl/mg)		
^{36}Cl space	3.45±0.13	3.82±0.03
^3H_2O space	4.16±0.11	5.33±0.10
[^{14}C]Sucrose	2.96±0.09	2.74±0.07
Calculated matrix space	1.20±0.10	2.59±0.06
Ion content of pellets (nequiv./mg)		
K^+	458±8	753±12
Na^+	90±17	92±3
P_i	54±2	186±7
Calculated matrix ion content (nequiv./mg)		
K^+	65±6	389±4
Na^+	70±4	73±3
P_i^{2-}	12±3	150±5
Cl^-	54±11	122±6
Matrix ion concentration (nequiv./μl)		
K^+	54	150
Na^+	58	28
P_i	10	58
Cl^-	45	47
Total matrix osmolarity (mOsM)	167	283

extensive accumulation of K^+ in the absence of phosphate, although the chloride space was found to be substantially greater than the sucrose space under the conditions of the experiment of Table 1. It is therefore likely either that a significant amount of Cl^- is bound to the outer surface of the mitochondria, or that a prior accumulation of a cation and phosphate, and a consequent swelling, is necessary if Cl^- is to penetrate the membrane.

An experiment designed to find out whether acetate would replace phosphate is shown in Table 2. Again there was a substantial increment in K^+ content and in sucrose-inaccessible space in the energized system, especially at the higher acetate concentration (40mM). The swelling of the matrix space is reasonably well explained on osmotic grounds. Thus, if the extra K^+ accumulated is divided by the gain in matrix space and an assumption is made that the ion accompanying the K^+ was in fact acetate, values of 464 and 388mOsM are obtained for the low- and high-acetate media respectively. These correspond to 272 and 312mOsM for the suspending media involved: there is thus better agreement with the high-acetate medium, where a larger uptake occurred. The low content of matrix K^+ in the incubation mixtures not including acetate may reflect replacement of endogenous K^+ by Na^+ added to the medium with the substrate glycerol phosphate. The high matrix Na^+ found in the experiment of Table 1 provides some evidence in favour of this suggestion. It may be concluded from these experiments that in state 4 respiration energy-linked uptake of K^+ occurs, that this uptake requires the presence of either phosphate or acetate, and that it does not occur in the presence of Cl^- only. Evidence will be presented below that the anion required must permeate as the undissociated acid; this requirement is not satisfied by anions such as Cl^- that penetrate as such, i.e. by anion uniport (see Chappell & Crofts, 1966; Mitchell & Moyle, 1969), under alkaline conditions.

Cation specificity

An investigation of the cations that are accumulated by state 4 blowfly mitochondria revealed that there is very little specificity; thus, results essentially similar to those presented for K^+, although less reproducible, were obtained in Na^+-containing media. Moreover, cations as large as choline were found to be accumulated. This was surprising, in view of the generally accepted finding that choline is a non-penetrant (see, e.g., Mitchell & Moyle, 1969). Subsequently it was found that uptake of these ions could be faithfully and less laboriously followed by

Table 2. *Facilitation of K^+ accumulation by acetate*

A basic medium was used comprising 0.12M-KCl, 2mM-potassium HEPES [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonate] buffer, pH7.1, 0.2μCi of [^{14}C]sucrose/ml, 2μCi of ^3H_2O/ml and 1mg of bovine serum albumin/ml. This was oxygen-saturated. To each 5ml portion of this medium were added, as indicated below, 25μmol of sodium DL-glycerol phosphate to the energized systems and 10μmol of KCN to the non-energized controls. In addition, the incubations contained the amount of potassium acetate shown below. Incubations were for 40s and were initiated by the addition of 0.25ml of mitochondrial suspension (21.8mg of protein/ml). Centrifugation and pellet extraction were as described in the Materials and Methods section. The temperature was 25°C. The values in the final column were obtained by subtracting from the K^+ content the product of the sucrose space and the concentration of K^+ in the suspending medium.

Concn. of acetate ...	None		15mM	40mM
Spaces (μl/mg)	+KCN	+Energy	+Energy	+Energy
H_2O	3.65	4.01	4.45	5.28
	3.82	4.15	4.52	5.15
Sucrose	2.67	3.00	3.04	3.00
	2.75	3.07	3.04	3.00
Calculated matrix	0.98	1.01	1.41	2.28
	1.07	1.08	1.48	2.15
Average matrix	1.03±0.04	1.04±0.03	1.44±0.04	2.22±0.06
K^+ content (nequiv./mg)	345	415	532	726
	348	389	518	680
Average	346	402	525	703
Calculated average matrix K^+ content (nequiv./mg)*	29	52	124	253
Calculated average matrix K^+ concentration (mM)	28	50	86	114

* Calculated as described in Table 1.

light-scattering changes, and a description of the effects of different large cations is given in a later section, in which that method is described. However, the unique behaviour of NH_4^+ requires special attention at this time. Tests with NH_4^+ showed that the uncoupled mitochondria contained more NH_4^+ and were more swollen than the state 4 mitochondria, i.e. the converse of the findings with other cations (Table 3). Parallel light-scattering experiments confirmed that a slow swelling occurred in NH_4^+-containing media on the addition of an uncoupling agent (Fig. 1). The rate of this swelling was significantly increased by raising the pH of the incubation medium from 7.1 to 7.9. This is consistent with the observation that the permeability to Cl^- is enhanced under these conditions (Azzi & Azzone, 1967; Brierley, 1970). Permeation of Cl^- in the experiment of Table 3 is indicated by the fact that insufficient phosphate enters to balance the K^+ found. The facilitation of the swelling in the presence of NH_4^+ by an uncoupling agent is then simply ascribed to the relief of a pH gradient built up by the formation of OH^- ions within the matrix owing to the entry of NH_4^+ as NH_3 (see Chappell & Crofts, 1966; Mitchell & Moyle, 1969). A scheme along these lines is shown in Figs. 2(*a*) and 2(*b*), which draw heavily on those authors' results. Control experiments (Fig. 1 and not shown) confirmed a prediction that would be made from this scheme, namely that uncoupling agents facilitate swelling in ammonium nitrate and ammonium chloride solutions (the latter at pH7.9) but have no effect on swelling in phosphate media. Respiration tended completely to abolish swelling in ammonium

Table 3. *Entry of NH_4^+ into coupled and uncoupled mitochondria*

Incubations were carried out in a volume of 5ml of a medium comprising 0.13M-NH_4Cl, 12mM-ammonium phosphate, pH7.2, 6mM-proline, 6mM-pyruvate, 0.8mg of bovine serum albumin/ml, 1μg of oligomycin/ml, 0.13μCi of [^{14}C]sucrose/ml and 1.6μCi of ^3H_2O/ml. Incubations were initiated by adding 0.2ml of mitochondrial suspension (24.4mg of protein/ml) and were ended by centrifugation after approx. 2min. Mean values are quoted for three coupled and three uncoupled incubations. The temperature was 25°C.

Condition	Non-energized	Energized
Spaces (μl/mg)		
H_2O	7.01	4.33
Sucrose	4.03	3.30
Calculated matrix space	2.98	1.03
Ion content (nequiv./mg)		
NH_4^+	1118±45	629±8
P_i	108±2	86±7
Calculated matrix ion content (nequiv./mg)*		
NH_4^+	547	160
P_i	62	47
Calculated matrix ion concentration (mM)		
NH_4^+	184	155
P_i	21	46
Ammonium phosphate osmolarity (mOsM)	205	201

* Calculated as described in Table 1.

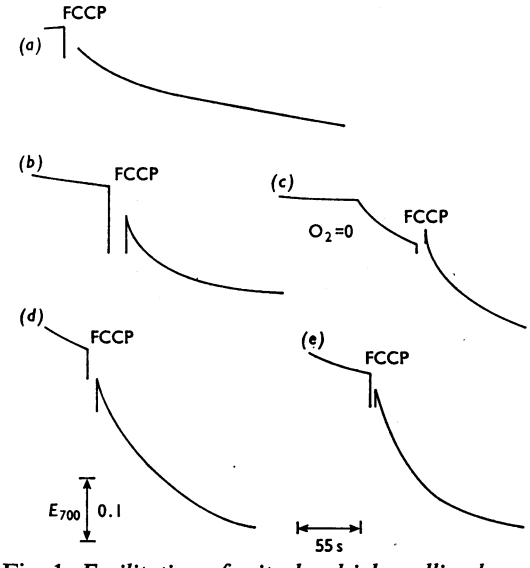

Fig. 1. *Facilitation of mitochondrial swelling by respiratory inhibitors and an uncoupling agent in NH_4Cl and NH_4NO_3 solutions*

E_{700} of mitochondrial suspensions in the following media was recorded: (*a*) an NH_4Cl plus ammonium phosphate medium identical with that used in the experiment of Table 3 (pH7.0); (*b*) the medium of experiment (a), with the pH adjusted to 7.9; (*c*) 150mM-NH_4Cl and 8mM-sodium DL-glycerol phosphate, pH7.9; (*d*) 150mM-NH_4Cl and 2mM-KCN, pH7.9; (*e*) 150mM-NH_4NO_3 and 2mM-KCN, pH7.1. Where indicated, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to 1μM. Experiments (*a*) and (*b*) employed one mitochondrial suspension; experiments (*c*), (*d*) and (*e*) another. The temperature was 25°C.

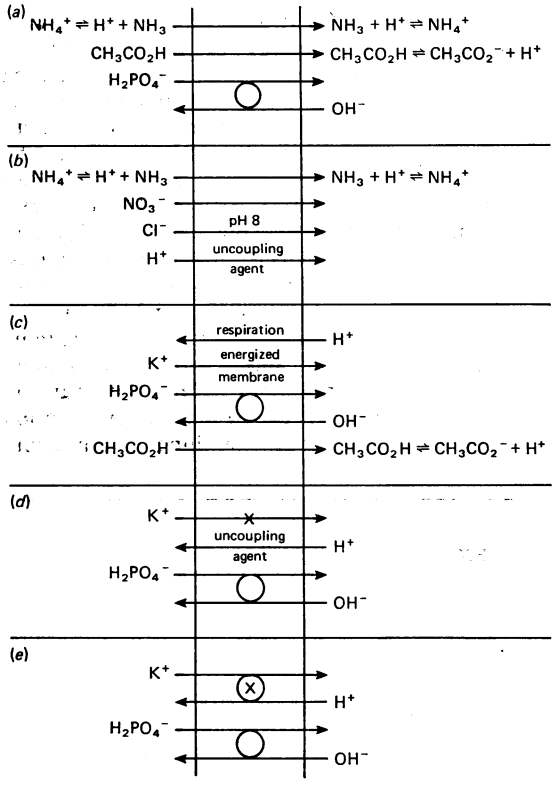

Fig. 2. *Model of cation and anion entry into blowfly flight-muscle mitochondria*

The indicated barrier to diffusion represents the inner mitochondrial membrane. A circle denotes a carrier intrinsic to the membrane, a cross the absence of the permeability thus negated. (*a*) and (*b*) suggest a mechanism of swelling in solutions of ammonium salts. Entry of ammonium acetate or ammonium phosphate (*a*) is electrically neutral, whereas entry of NH_4NO_3 or NH_4Cl (pH 8) leads to the formation of a membrane potential and only occurs in the presence of an uncoupling agent. (*c*) illustrates the suggested involvement of a K^+ uniporter in swelling in potassium phosphate and potassium acetate solutions, in the presence of an oxidizable substrate. (*d*) and (*e*) explain the evidence for ruling out a K^+ uniporter and a K^+/H^+ antiporter, respectively, in the non-energized state. Swelling occurred under the conditions of (*a*), (*b*) and (*c*) only. It would have occurred in (*d*) and (*e*) had the K^+ carrier marked with a cross been present.

nitrate and ammonium chloride solutions, i.e. to decrease it even below the rate obtained in the absence of uncoupling agent. This could be attributed to an inhibition of NH_4^+ entry by alkalinity of

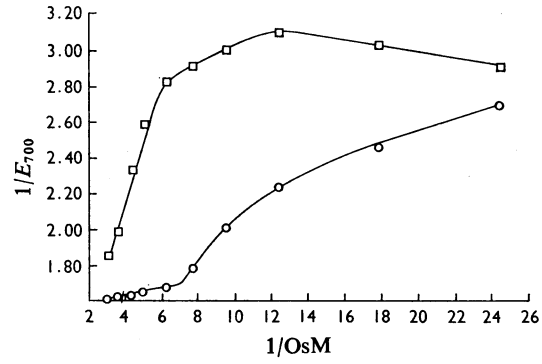

Fig. 3. *Limited osmotic response of blowfly flight-muscle mitochondria in potassium and ammonium chloride solutions*

E_{700} was measured 10min after mitochondria had been added to solutions of the osmolarities shown. All solutions contained 5mM-tris–HCl, pH 7.4, 1mM-EGTA and 1μg of antimycin A/ml. ○, KCl; □, NH_4Cl.

the matrix during respiration (Mitchell, 1965). It seems, then, that the cation uptake phenomena described in this paper cannot be fruitfully studied in NH_4^+-containing media. It is argued in the Discussion section that the different behaviour in K^+- and NH_4^+-containing solutions reflects the electrically neutral character of NH_4^+ penetration, but the electrogenic or electrophoretic character of K^+ entry.

Lack of penetration of respiration-inhibited or uncoupled mitochondria by alkali-metal cations

The results just described are difficult to reconcile with the generally accepted view that K^+ hardly penetrates the mitochondrial membrane at all in the absence of ionophorous antibiotics (see, e.g., Gamble, 1957; Amoore & Bartley, 1958; Amoore, 1960; Rottenberg & Solomon, 1965; Christie *et al.*, 1965; Harris *et al.*, 1967) or enters in sluggish antiport for H^+ (Mitchell & Moyle, 1967, 1969). Repetition of the swelling experiments described by Chappell & Crofts (1966) as a criterion of mitochondrial permeability revealed that respiration-inhibited blowfly flight-muscle mitochondria do not swell in iso-osmotic potassium chloride, potassium acetate or tetramethylammonium acetate solutions, whereas they swell with great rapidity in iso-osmotic ammonium acetate or phosphate solutions, supporting the idea that the K^+ ion is indeed a non-penetrant when mitochondria are not respiring.

In ammonium chloride solutions they swell slowly, even at pH 7.8, in the absence of uncoupling agents. Fig. 3 shows that these mitochondria behave as

osmometers in potassium chloride solutions at more than 60mM; in ammonium chloride there is some osmotic response, but there is a slow swelling. Comparison of the two curves suggests that it must be permeability to K^+ that limits swelling in potassium chloride and that a permeability must be elicited when the membrane is stretched, for instance by using suspending media at less than 60mM. From this plot one can argue that there is neither a significantly rapid uniport of K^+ nor K^+/H^+ antiport in these mitochondria when respiration is inhibited. Neither H^+ permeability nor Cl^- permeability can limit the rate of swelling in potassium chloride, in view of the response to ammonium chloride.

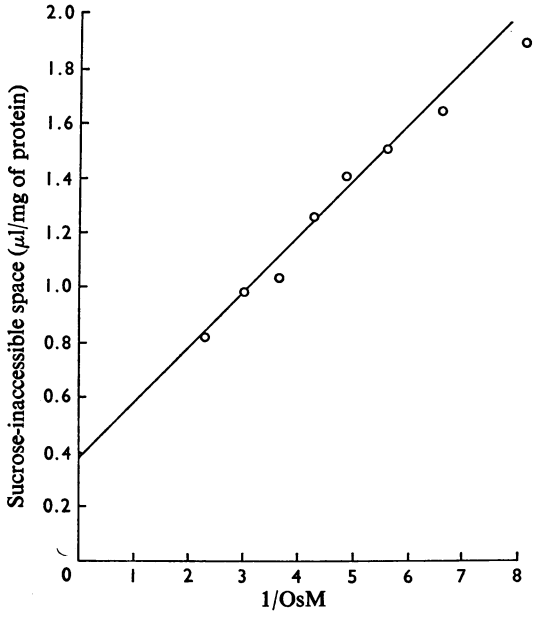

Fig. 4. *Osmotic behaviour of blowfly flight-muscle mitochondria in KCl solutions, evaluated by the [^{14}C]sucrose-inaccessible-space method*

Portions (0.18ml) of a mitochondrial suspension (20.4mg of protein/ml) were added to tubes containing 3ml of KCl solutions of the concentration indicated. The various concentrations were achieved by mixing (1) 0.2M-KCl, 5mM-potassium HEPES buffer, pH7.2, 2mM-KCN and (2) 5mM-potassium HEPES buffer, pH7.2, 2mM-KCN solutions in the appropriate proportions. Each solution contained 0.72μCi of ^3H_2O/ml and 0.13μCi of [^{14}C]sucrose/ml and the two solutions were pH-matched. Incubations were for 2min at 25°C and were ended by centrifugation. Pellets were extracted and counted for radioactivity as described in the Materials and Methods section. The sucrose-inaccessible space was calculated and is plotted against the reciprocal of the osmolarity of the medium. Analysis of the mitochondria used in this experiment by flame photometry indicated a high endogenous K^+ content (110nequiv./mg).

An investigation of the osmotic behaviour of non-respiring blowfly flight-muscle mitochondria in potassium chloride solutions by using the spaces method described earlier (Fig. 4) confirmed the conclusion from the light-scattering experiment: these mitochondria behave as osmometers in potassium chloride solutions of concentrations over 60mM and are thus impermeant to K^+. Moreover, the volume of the osmotically sensitive compartment can be measured with some precision, contrary to work of Klingenberg & Buchholz (1970). Thus the values of matrix volume plotted in Fig. 4 are the differences obtained by subtracting the [^{14}C]sucrose space from the ^3H_2O space for each individual pellet. This plot confirms that the matrix volume of respiration-inhibited blowfly flight-muscle mitochondria is 1μl/mg of protein at 300mOsM, approximately the value arrived at previously (from results in Tables 1 and 2). The osmotic dead-space, which is largely composed, according to Pfaff *et al.* (1968), of the hydration sphere of proteins, was found to be 0.37μl/mg.

Effect of energy on the permeability to cations

The preceding work suggested that the permeability of the membrane to cations was enhanced during the coupled oxidation of substrate in state 4. The accumulation experiments had involved predominantly Cl^--containing media, as this was the system used as a routine in polarographic experiments. However, for the examination of cation permeability there were clearly advantages in using media in which all of the anion was capable of rapid penetration. Thus, experiments were done in iso-osmotic acetate and phosphate solutions of a number of different cations (Fig. 5). In each case, swelling was very slow until substrate (DL-glycerol phosphate) was added, whereupon it was accelerated by up to 100-fold. Addition of uncoupling agents during the slow initial phase of the swelling gave no enhancement in rate, and prevented any subsequent enhancement on addition of substrate. The most notable feature of these experiments was that in the presence of substrate there was a high rate of swelling even when the cation present was as large as tris or ornithine. The only exceptions to this were the responses in calcium acetate and magnesium acetate solutions, where the addition of substrate resulted in a small and instantaneous decrease in light-scattering, followed by a much slower phase. Certainly, during this latter phase these salts were providing some osmotic support, a conclusion consistent with the results reported by Carafoli *et al.* (1971) on the impermeability of these mito-

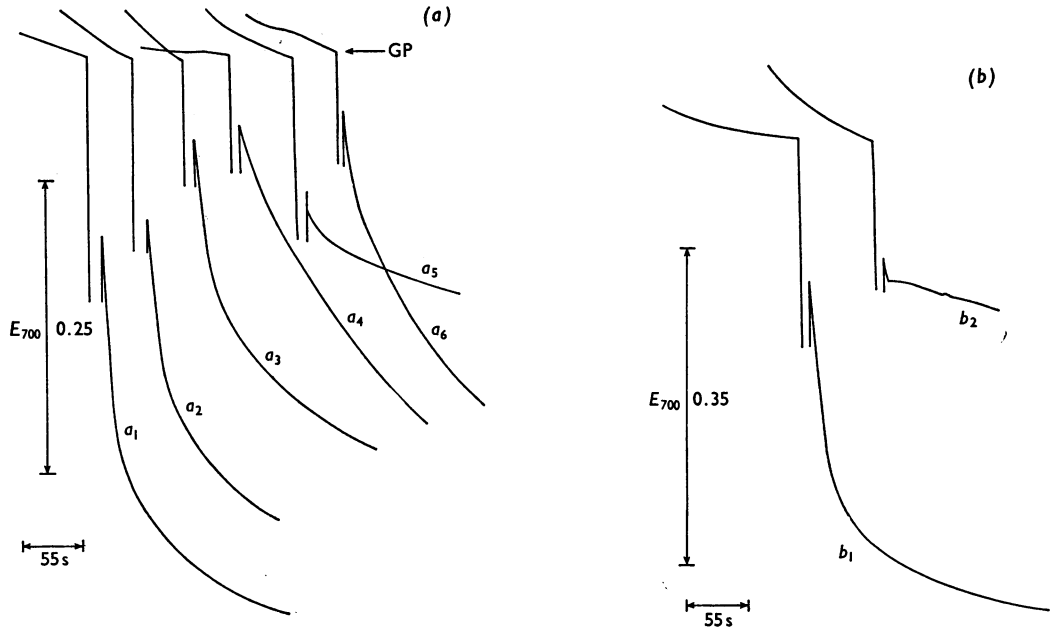

Fig. 5. *Enhancement by* DL-*glycerol phosphate of swelling in iso-osmotic acetates and potassium phosphate*

Mitochondria were added to the following solutions, all pH7.2 and containing 1 μM-rotenone. The E_{700} was measured at 25°C. Where indicated, tris–DL-glycerol phosphate (GP) was added, to 8mM. (*a*) The original E_{700} of the suspension was 0.82; (1) 0.16M-potassium acetate; (2) 0.16M-sodium acetate; (3) 0.16M-tris acetate; (4) 0.16M-ornithine acetate; (5) 0.106M-magnesium acetate; (6) 0.128M-potassium phosphate. (*b*) A different mitochondrial suspension was used: (1) 0.16M-potassium acetate; (2) 0.106M-calcium acetate.

chondria to Ca^{2+}. These results resemble those obtained with heart mitochondria suspended in acetate solutions (Brierley *et al.*, 1968; Blondin & Green, 1969), and extend the findings of these authors to phosphate solutions, where the anion also enters effectively as the acid (Mitchell & Moyle, 1969).

Replacement of the substrate with pyruvate and proline (the latter to provide oxaloacetate; Childress & Sacktor, 1966) gave rise to a much lower rate of swelling. Addition of oligomycin enhanced this rate (Fig. 6) and a subsequent addition of ADP increased it still more. ADP under these conditions has the role of activating the NAD-linked isocitrate dehydrogenase (EC 1.1.1.41) and thereby increasing the reduction of NAD. These facts suggested that it might be the redox state of the respiratory carriers that was responsible for the increased permeation, rather than the energy state of the mitochondrion, as the carriers are notably reduced by glycerol phosphate in state 4 and by pyruvate in the presence of oligomycin and ADP (Hansford, 1968). However, an experiment with glycerol phosphate and KCN, which would give a high degree of reduction (except of nicotinamide nucleotide) showed no facilitation of swelling. Inclusion of ADP in iso-osmotic potassium phosphate media or in potassium acetate media containing 2mM-potassium phosphate prevented any swelling on the addition of glycerol phosphate, until the ADP had been phosphorylated (Fig. 7). Phosphorylation thus prevents this massive influx of cation and acetate or phosphate. Addition of valinomycin accelerates swelling in the presence of substrate, and elicits swelling when added during phosphorylation (Figs. 7 and 8). These findings, presented in a quantitative fashion in Table 4, are taken to indicate that energization does indeed increase the permeability of the membrane (see the Discussion section).

Discussion

Oxygen-electrode experiments with blowfly flight-muscle mitochondria show that respiratory control with pyruvate as substrate is optimum in salt media containing 20mM-phosphate (Hansford, 1968). The present study shows that under these conditions there is a massive influx of cation and phosphate during

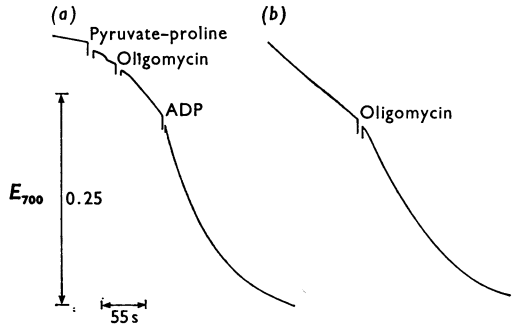

Fig. 6. *Enhancement by pyruvate oxidation of mitochondrial swelling in iso-osmotic potassium phosphate solutions*

Mitochondria were suspended in 0.128 M-potassium phosphate, pH 7.2, at 25°C. The record is that of change in E_{700}, the value at the beginning of the experiment being 0.8. In (*a*), pyruvate (2 mM), proline (5 mM), oligomycin (1 μg/ml) and ADP (2 mM) were added as indicated. In (*b*), the medium contained pyruvate and proline initially, and oligomycin was added as indicated.

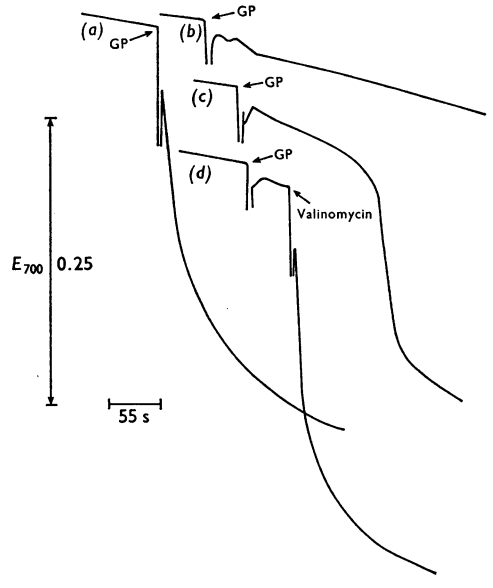

Fig. 7. *Inhibition of mitochondrial swelling in iso-osmotic potassium phosphate solutions by the phosphorylation of ADP*

Mitochondria were suspended in 0.128 M-potassium phosphate, pH 7.2, in the presence of 1 μM-rotenone. (*a*) No further component of the medium; (*b*) 10 μmol of ADP present initially; (*c*) 2 μmol of ADP present initially; (*d*) 10 μmol of ADP present initially. In each case, 15 μmol of sodium DL-glycerol phosphate (GP) was added as indicated. In (*d*), 5 μl of a 6.5 μg/ml solution of valinomycin was added, as indicated. The total volume in each case was 2.55 ml and the temperature 25°C. In (*c*), the point of inflexion of the curve corresponds to the completion of phosphorylation of the ADP present. The recording is that of E_{700}, the initial value being 0.757.

state 4 respiration and a consequent enlargement of the matrix. Clearly, this has no very deleterious effect on mitochondrial performance. It differs, apparently, from the 'phosphate swelling' in liver mitochondria investigated by Harris & van Dam (1968), in that it is the matrix and not the cristal space which expands. This result is not surprising in that selective permeability is normally thought to involve the inner membrane.

Calculations on the osmolarity of the salt taken up under these conditions show that it is equal to that of the suspending medium to a first approximation, a prediction of the osmotic model of swelling (Tedeschi, 1961; Chappell & Crofts, 1966; Rasmussen *et al.*, 1965). This is surprising, as these mitochondria possess a denser array of cristae than those from other tissues (Smith, 1963), with a correspondingly ordered matrix. If ion adsorption were to play a role in this sort of experiment, it would surely be observed in these mitochondria, because of the large surface area. To this extent, these studies widen the validity of the osmotic model which was first proposed for mitochondria of a very different structure. The actual size of the matrix compartment of blowfly mitochondria is very similar to that obtained for the mannitol-impermeable space of ox heart mitochondria by Hunter & Brierley (1969) and somewhat larger than that found for the sucrose-impermeable space of liver mitochondria by Bartley (1961) and Harris & van Dam (1968) (but cf. larger values obtained for liver by Pfaff *et al.*, 1968). Comparison along these lines may not be very meaningful, however, as they are on a protein basis, and the proportions of the total protein contributed by matrix and membrane vary considerably among these mitochondrial types.

The difference between the penetration of these mitochondria by cations in the presence and absence of energy is best explained by the induction of a non-specific cation permeability in the energized or state 4 condition; Brierley *et al.* (1968) and Blondin & Green (1969) suggested an increased permeability to cations from experiments similar to those shown in Fig. 5. However, an increase in net permeation under these conditions is not an adequate criterion of an increased permeability. Thus, if cations move down an electrochemical gradient, as suggested by the chemiosmotic

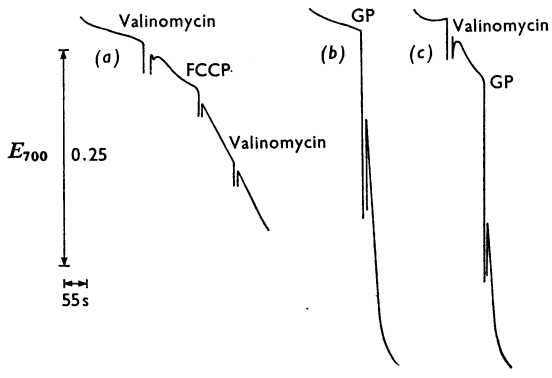

Fig. 8. *Effect of valinomycin on the rate of mitochondrial swelling in energized and non-energized states*

Mitochondria were incubated in 0.128M-potassium phosphate, pH 7.2, containing 1μM-rotenone. Where indicated, 13ng of valinomycin (*a*), 32.5ng of valinomycin (*c*), 2.5μmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 15μmol of sodium DL-glycerol phosphate (GP) were added.

hypothesis (Mitchell, 1965), addition of substrate will increase the rate of cation permeation by adding an electrical component to the concentration component already present. Clearly, if there is absolutely no entry in the absence of substrate then energization must be inducing a permeability change as well as superimposing a membrane potential, but this condition is not fully met (Fig. 5). However, results with valinomycin presented here do allow the implication of a permeability change. Thus, in the experiment of Fig. 7, where valinomycin greatly facilitated swelling in state 3, it is most unlikely that it was acting to increase the electrochemical gradient, much more likely that it was increasing the permeability of the membrane, the effect normally attributed to this antibiotic (Chappell & Crofts, 1965). A less well-defined effect of valinomycin in achieving a marshalling of the energy supplies of the mitochondrion into use in cation uptake (Harris *et al.*, 1966) might, however, invalidate this argument. When rates of swelling under these conditions are compared (Table 4) it becomes apparent that the membrane potential in state 3 must be large, indeed appears not greatly diminished with respect to state 4 and that the lack of swelling before the addition of valinomycin can only reflect an impermeability to K^+.

It thus seems that the membrane of blowfly flight-muscle mitochondria is permeable to cations in state 4, but not in state 3 or in the absence of energy (i.e. oxidizable substrate). Moreover, this permeability involves the movement of cations alone, not obligatorily linked to that of any other ion. These conclusions follow from several observations. First, the rate of swelling in iso-osmotic solutions of potassium phosphate and potassium acetate is extremely low in the absence of energy. By contrast, swelling in the corresponding ammonium salts is both rapid and extensive. These results rule out rapid antiport of K^+ and H^+ in the non-energized state (Fig. 2*e*). Moreover, addition of an uncoupling agent in no way facilitates swelling, as would happen if K^+ were transported by a uniporter, with the consequent formation of a membrane potential (Fig. 2*d*). Secondly, on addition of a respiratory substrate a massive swelling occurs in iso-osmotic solutions of the acetates and phosphates of a number of cations (Fig. 5). In comparison, the swelling seen in corresponding chloride solutions on the addition of substrate is very slight (not shown) even when the experiment is carried out at pH8, to guarantee permeability of the mitochondrial membrane to Cl^-. As the entry of phosphate or acetate is an electrically neutral process (Chappell & Crofts, 1966; Mitchell & Moyle, 1969) as shown in Fig. 2, the entry of cations during respiration must also be neutral for a large swelling to occur. If it is accepted that respiration *per se* leads to the expulsion of H^+ ions from the mitochondrion, and is hence an electrogenic process, then cation movement in the opposite direction must be electrophoretic (Fig. 2). On this reasoning, the lack of swelling in potassium chloride (pH8) in the presence of energy would reflect the scant intrinsic permeability of the membrane to H^+ ions.

The induction of a permeability to cations which seems to occur when an oxidizable substrate is added to these mitochondria in some ways resembles the effect of the ionophorous antibiotic valinomycin

Table 4. *Effect of valinomycin on energized and non-energized swelling in iso-osmotic potassium phosphate*

Mitochondria (approx. 1mg of protein) were suspended in 2.5ml of 0.12M-potassium phosphate, pH7.2, and E_{700} was recorded with a Unicam SP.800 spectrophotometer. The temperature was 25°C. Glycerol phosphate, ADP and valinomycin were added as detailed below. The rate was measured as the rate of decrease of E_{700} with time.

DL-Glycerol phosphate (mM)	ADP (mM)	Valinomycin (ng/ml)	Rate (arbitrary units)
—	—	—	0.2
10	—	—	60
10	1	—	0.4
10	1	10	73
10	—	10	80
—	—	10	11

(Moore & Pressman, 1964; Pressman, 1965; Cockrell *et al.*, 1966; Harris *et al.*, 1966; Azzi & Azzone, 1966). It differs, however, in that there is little discrimination between cations of different sizes (Fig. 5) and in that a subsequent addition of valinomycin gives a further enhancement (Fig. 8).

The experimental results obtained in polarographic media (Table 1, Fig. 1) can be explained on the basis of an increased permeability to whatever cation was present, including those as large as choline, and a consequent uptake of cation and phosphate. The magnitude of this would be somewhat limited by the concentration of phosphate present, but once a certain swelling had been achieved the membrane would have become permeable to Cl^- (at neutral pH), as shown in Fig. 3. Some penetration of Cl^- could then occur (Table 1), though this would be limited by the H^+-ion permeability of the membrane.

How does this cation permeability in state 4 compare with that found in other mitochondria, and what is its bearing on oxidative phosphorylation? The rate of the uptake is high. The values for net accumulation (Tables 1 and 2) will necessarily be minimal values for uptake (Harris *et al.*, 1966). They are nevertheless severalfold larger than the rates reported by Harris *et al.* (1966) for K^+ uptake by liver mitochondria, non-facilitated by valinomycin, and are equally in excess of the ^{42}K-exchange rates reported by the same authors. In this study (Harris *et al.*, 1966) the accumulation was measured in the presence of a respiratory substrate, acetate as a penetrant anion, and 10mM-K^+. The metabolic status of the mitochondria used for the exchange study was not clear. Another study (Harris *et al.*, 1967) reported rather lower rates of K^+ entry, in the absence of valinomycin. Therefore the high rate of entry of cations into blowfly flight-muscle mitochondria may reflect more the very much greater inner-membrane area in these mitochondria than an increased leakiness of the membrane per unit area. This would be consistent with findings that respiration is tightly coupled under conditions of ion uptake. It is clear from Fig. 7 that phosphorylation can compete effectively with ion uptake for energy reserves. This could be equally well explained as competition for a high-energy intermediate (Slater, 1953, 1958), an energized carrier (Massari & Azzone, 1970), or an electrochemical gradient (Mitchell, 1965). In state 4, the cation uptake should impose an energy demand and lead to an increased respiratory rate. This is found to occur. The relevant experiments are in practice difficult to do, as the oxidation of pyruvate, which is subject to tight respiratory control (respiratory control ratios of 20–30), is also sensitive to ADP and phosphate as allosteric effectors, which makes the effects of addition of phosphate difficult to interpret. These difficulties do not apply to the study of glycerol phosphate oxidation, which is, however, relatively loosely coupled. With the latter substance as substrate the addition of phosphate to a concentration of 5mM stimulated the state 4 rate from 0.401 to 0.535μg-atoms of oxygen/min per mg of protein for about 30s, after which the lower rate was again obtained. A subsequent addition of ADP elicited the state 3 rate of 1.23μg-atoms of oxygen/min per mg of protein. Addition of phosphate to a higher concentration increased the stimulated rate slightly, but tended to decrease subsequent response to ADP. Comparison with centrifugation experiments carried out under similar conditions (Tables 1 and 2) would suggest that during this period of stimulated respiration the ratio of K^+ taken up to extra oxygen consumed was very approximately 2. The accumulation of K^+ thus represents a drain in the supply of respiratory energy. However, in the presence of 5mM-phosphate the stimulated respiration returns to normal after cation accumulation; the latter condition is presumably that obtaining in the intact cell *in vivo*.

The basic question may be asked as to whether the increased penetration of cations in state 4 and failure to penetrate in state 3 is due to a change in physical state of the membrane so that there is a change in permeability per unit area or due to an increase in the exposed area of inner membrane consequent to unfolding of cristae, with no change in the intrinsic permeability coefficient. Electron micrographs of liver mitochondria in state 3 and state 4 have revealed very large changes in the matrix volume and in the exposed area of inner membrane in 'orthodox' or state 4 mitochondria (Hackenbrock, 1966, 1968). It will be worthwhile to determine whether corresponding changes in the morphology of fly flight-muscle mitochondria occur, and can be visualized by using the electron microscope.

References

Amoore, J. E. (1960) *Biochem. J.* **76**, 438

Amoore, J. E. & Bartley, W. (1958) *Biochem. J.* **69**, 223

Azzi, A. & Azzone, G. F. (1966) *Biochim. Biophys. Acta* **113**, 445

Azzi, A. & Azzone, G. F. (1967) *Biochim. Biophys. Acta* **131**, 468.

Bartley, W. (1961) *Biochem. J.* **80**, 46

Bartley, W. & Davis, R. E. (1954) *Biochem. J.* **57**, 37

Berenblum, J. & Chain, E. (1938) *Biochem. J.* **32**, 295

Blondin, G. A. & Green, D. E. (1969) *Arch. Biochem. Biophys.* **132**, 509

Bray, G. A. (1960) *Anal. Biochem.* **1**, 279

Brierley, G. P. (1970) *Biochemistry* **9**, 697.

Brierley, G. P., Settlemire, C. T. & Knight, V. A. (1968) *Arch. Biochem. Biophys.* **126**, 276

Carafoli, E., Hansford, R. G., Sacktor, B. & Lehninger, A. L. (1971) *J. Biol. Chem.* **246**, 964.

Caswell, A. H. (1969) *J. Membrane Biol.* **1**, 53

Chance, B. & Williams, G. R. (1955) *J. Biol. Chem.* **217**, 409

Chappell, J. B. (1954) Ph.D. Dissertation, University of Cambridge

Chappell, J. B. & Crofts, A. R. (1965) *Biochem. J.* **95**, 393
Chappell, J. B. & Crofts, A. R. (1966) *Regul. Metab. Processes Mitochondria, Proc. Symp.* 293
Childress, C. C. & Sacktor, B. (1966) *Science* **154**, 268
Christie, G. S., Ahmed, K., McLean, A. E. M. & Judah, J. D. (1965) *Biochim. Biophys. Acta* **94**, 432
Cockrell, R., Harris, E. J. & Pressman, B. C. (1966) *Biochemistry* **5**, 2326
Gamble, J. L., Jr. (1957) *J. Biol. Chem.* **228**, 955
Hackenbrock, C. R. (1966) *J. Cell Biol.* **30**, 269
Hackenbrock, C. R. (1968) *J. Cell Biol.* **37**, 345
Hansford, R. G. (1968) Ph.D. Dissertation, University of Bristol
Harris, E. J. & van Dam, K. (1968) *Biochem. J.* **106**, 759
Harris, E. J., Cockrell, R. & Pressman, B. C. (1966) *Biochem. J.* **99**, 200
Harris, E. J., Catlin, G. & Pressman, B. C. (1967) *Biochemistry* **6**, 1360
Hunter, G. R. & Brierley, G. P. (1969) *Biochim. Biophys. Acta* **180**, 68
Klingenberg, M. & Buchholz, M. (1970) *Eur. J. Biochem.* **13**, 247
Massari, S. & Azzone, G. F. (1970) *Eur. J. Biochem.* **12**, 310
Mitchell, P. (1965) *Biol. Rev. Cambridge Phil. Soc.* **41**, 445
Mitchell, P. (1967) *Advan. Enzymol. Relat. Areas Mol. Biol.* **29**, 33
Mitchell, P. & Moyle, J. (1967) *Biochem. J.* **105**, 1147
Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **9**, 149
Moore, C. & Pressman, B. C. (1964) *Biochem. Biophys. Res. Commun.* **15**, 562
Pfaff, E., Klingenberg, M., Ritt, E. & Vogell, W. (1968) *Eur. J. Biochem.* **5**, 222
Pressman, B. C. (1965) *Proc. Nat. Acad. Sci. U.S.* **53**, 1076
Price, G. M. & Lewis, S. E. (1959) *Biochem. J.* **71**, 176
Rasmussen, H., Chance, B. & Ogata, E. (1965) *Proc. Nat. Acad. Sci. U.S.* **53**, 1069
Rosen, H. (1957) *Arch. Biochem. Biophys.* **67**, 10
Rottenberg, H. & Solomon, A. K. (1965) *Biochem. Biophys. Res. Commun.* **20**, 85
Slater, E. C. (1953) *Nature (London)* **172**, 975
Slater, E. C. (1958) *Rev. Pure Appl. Chem.* **8**, 221
Smith, D. S. (1963) *J. Cell Biol.* **19**, 115
Spector, W. G. (1953) *Proc. Roy. Soc. Ser. B* **141**, 268
Tedeschi, H. (1961) *Biochim. Biophys. Acta* **46**, 159