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CONVERSION OF BIOMEMBRANE-PRODUCED ENERGY INTO ELECTRIC FORM

II. INTACT MITOCHONDRIA

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SUMMARY

The transport of synthetic ions penetrating across intact mitochondrial membranes has been investigated. It is shown that anions of phenyl dicarbaundecaborane (PCB^-) are extruded from mitochondria on transition to the energized state. Discharge of the energized state is accompanied by movement of the extruded anions back into the mitochondria.

The penetrating cations dibenzyl dimethyl ammonium (DDA^+), tetrabutyl ammonium and triphenyl methyl phosphonium, when added to liver or heart mitochondria in the presence of oxidizable substrates or ATP, bring about the same responses that accompany the active transport of natural penetrating cations Ca^{2+} or K^+ in the presence of valinomycin, *i.e.* acidification of the incubation mixture, a transient increase in ATPase and oxidation rate in State 4, cyclic oxidation of NAD(P)H reduced by succinate and swelling of the mitochondrial matrix. The latter process requires the addition of inorganic phosphate.

DDA^+-induced swelling is found to be supported by both ATP hydrolysis and respiratory chain electron transfer from substrates to oxygen or to ferricyanide.

All effects of penetrating cations in mitochondria are potentiated by the addition of small amounts of the penetrating anions, PCB^- or tetraphenyl boron, which increase the permeability of the membrane for the cations under study.

The data obtained confirm the conclusion that it is the electric field (negative inside the mitochondria) which is the motive force for the transport of penetrating ions across the mitochondrial membrane.

INTRODUCTION

In the previous paper, the phenomenon of accumulation of anions penetrating submitochondrial particles was described[1]. It was found that the energy-dependent

Abbreviations: PCB^-, phenyl dicarbaundecaborane; DDA^+, *N,N*-dibenzyl *N,N*-dimethyl ammonium cation; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; TTFB, tetrachlorotrifluoromethyl benzimidazole.

mechanism of anion transport in the sonicated particles was rather nonspecific for the structure of penetrating anions; penetrating cations were not accumulated. It was concluded that energy-dependent accumulation of penetrating anions by particles is due to the movement of these anions in the electric field orientated across the membrane of the particle so that the *'plus'* is inside and the *'minus'* outside. This paper summarizes the results of a study on the transport of synthetic penetrating ions across the membrane of intact mitochondria which have the opposite orientation.

METHODS

Preparation of mitochondria

Beef heart mitochondria were prepared by the method of CRANE *et al.*[2]. For the isolation of rat liver and rabbit heart mitochondria, a solution of sucrose (0.3 M) and EDTA (0.01 M) at pH 7.5 was used. Nuclei and cell debris were removed by centrifugation for 10 min at 600 × *g*. Mitochondria were sedimented at 7000 × *g* (liver) or 16000 × *g* (heart) for 15 min. The sedimented mitochondria were suspended in the sucrose–EDTA solution. In some experiments the method of MOSOLOVA *et al.*[3], including four washings of mitochondria, was used.

The kinetics of mitochondrial swelling were measured spectrophotometrically at 520 nm.

Electron microscopic studies were carried out using a Hitachi HU-11B microscope. Mitochondria were fixed with 5 % glutaraldehyde and treated with OsO_4, alcohols, uranyl acetate and epoxide resin 812. Thin sections of mitochondria were prepared with an LKB-4800 ultramicrotome.

For other methods see the previous paper[1].

RESULTS AND DISCUSSION

Effect of mitochondria on the concentration of penetrating anions in solution

Fig. 1 demonstrates the changes in phenyl dicarbaundecaborane anion (PCB^-) concentration after addition of rat liver mitochondria. It is seen that mitochondria, like phospholipid micelles and submitochondiral particles (see Fig. 3 in the previous paper[1]) absorb the penetrating anions. This process proved to be energy-independent due to the passive uptake of the anions by the lipid material.

Addition of the oxidizable substrate (succinate) induces a small but measurable anion extrusion. Inhibition of respiration by antimycin results in the uptake of a portion of the PCB^- anions that had been extruded in the energized state. Addition of ATP induces another PCB^- efflux, and the subsequent addition of the uncoupler, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone (FCCP), causes PCB^- influx. Thus, transitions between energized and deenergized states in mitochondria are accompanied by movements of PCB^- in the opposite direction to that previously observed in sonicated submitochondrial particles[1]. The amplitude of the changes in PCB^- concentration in the course of transition between the energized and deenergized states in samples with mitochodnria, was always much smaller than in the experiments with sonicated particles. This is not unexpected since anions are pumped out of the intramitochondrial space, which represents only a very small fraction of

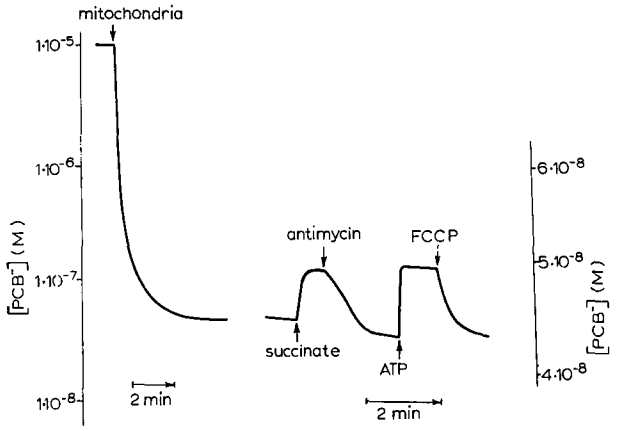


Fig. 1. Effect of rat liver mitochondria on PCB^- concentration in solution. Incubation mixture: 0.25 M sucrose, 0.05 M Tris buffer (pH 7.5), $6 \cdot 10^{-3}$ M $MgSO_4$, $3 \cdot 10^{-5}$ M rotenone, 3.3 mg/ml mitochondrial protein. Additions: 0.01 M succinate, $8 \cdot 10^{-7}$ M antimycin A, $2 \cdot 10^{-3}$ M ATP, $3 \cdot 10^{-7}$ M FCCP.

the total volume of the sample. These relationships could be responsible for some of the difficulties in demonstrating anion extrusion by mitochondria, since even a small contamination by submitochondrial particles would obscure the mitochondrial transport reactions. For this reason the mitochondria were washed four times in these experiments[3].

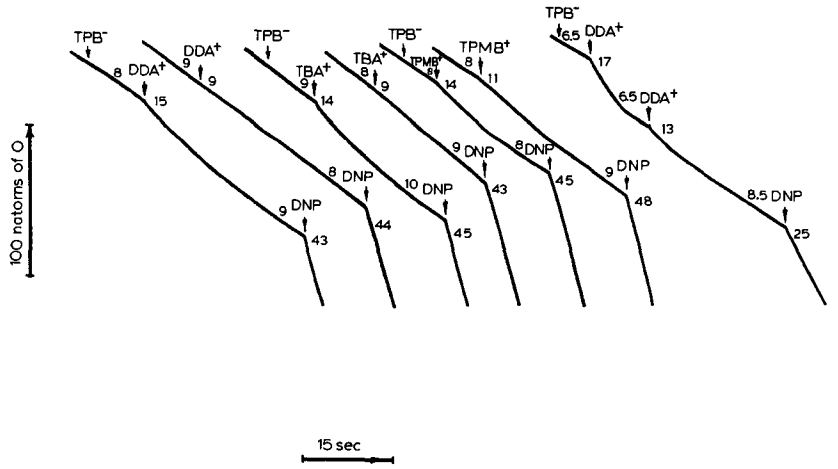
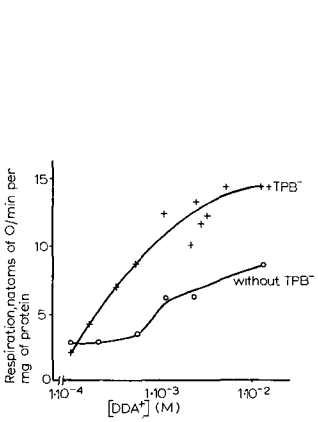


Fig. 2. The effect of penetrating ions on the respiration of rat liver mitochondria. Reaction mixture: 0.3 M sucrose, 0.05 M Tris buffer (pH 7.5), 15 mg/ml mitochondrial protein, $1.7 \cdot 10^{-2}$ M succinate, $1 \cdot 10^{-6}$ M rotenone. Additions: $3.5 \cdot 10^{-4}$ M DDA^+, $1.2 \cdot 10^{-3}$ M triphenyl methyl phosphonium ($TPMP^+$), $3.5 \cdot 10^{-4}$ M tetrabutyl ammonium (TBA^+), $2.3 \cdot 10^{-5}$ M tetraphenyl boron (TPB^-), $1.2 \cdot 10^{-4}$ M 2,4-dinitrophenol (DNP). In the last sample the concentrations of DDA^+ were $6 \cdot 10^{-4}$ M (first addition) and $1.1 \cdot 10^{-3}$ M (second addition). Figures close to curves: rate of respiration in natoms of O/min per mg of mitochondrial protein.

Effects of synthetic penetrating cations on mitochondrial electron transfer

While the cations of *N*,*N*-dibenzyl *N*,*N*-dimethyl ammonium (DDA^+), triphenyl methyl phosphonium and tetrabutyl ammonium did not affect the functions of submitochondrial particles[1], they induced a number of rather characteristic responses in mitochondria, acting in a way similar to Ca^{2+} (or K^+ in the presence of valinomycin). These responses suggest that the penetrating cations are engaged in some energy-requiring process in the mitochondria. As is shown in Fig. 2, addition of penetrating cations increases the rate of oxygen uptake in State 4. The effect of cations on respiration is greatly potentiated by the addition of small amounts of penetrating anions such as tetraphenyl boron (or PCB^-). In Fig. 3, the rate of oxidation is plotted against the concentration of DDA^+ in the presence and in the absence of tetraphenyl boron. It is seen that smaller concentrations of DDA^+ cations are needed to increase the oxidation rate in State 4 when tetraphenyl boron anions are present in the medium. This effect is in agreement with that observed in the experiments with phospholipid membranes when low concentrations of tetraphenyl boron enhance the permeability of the membrane to DDA^+ cations (see ref. 1, Fig. 1). In Fig. 4 the effect of DDA^+ is shown as a function of tetraphenyl boron concentration.

It should be noted that the increase in respiration rate induced by synthetic penetrating cations (as well as in the case of natural cations) is transient, being followed by inhibition of respiration up to a level close to that in State 4. A second addition of the cations induces a new cyclic change in the respiration rate. Subsequent addition of 2,4-dinitrophenol leads to a steady increase in the rate of oxygen consumption (see Fig. 2).



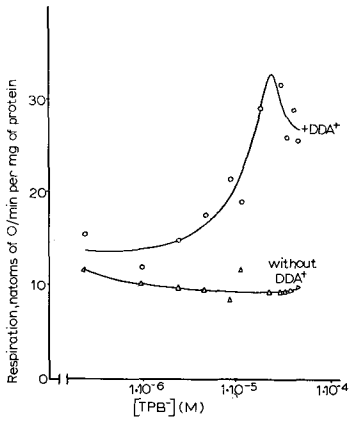


Fig. 3. The effect of DDA^+ on mitochondrial respiration as a function of DDA^+ concentration. Incubation mixture as described in Fig. 2. The concentration of tetraphenyl boron (TPB^-) was $1.8 \cdot 10^{-5}$ M.

Fig. 4. The effect of tetraphenyl boron (TPB^-) on mitochondrial respiration in the presence and in the absence of DDA^+. Incubation mixture as described in Fig. 2. The concentration of DDA^+ was $1.2 \cdot 10^{-3}$ M.

The transient stimulation of respiration upon the addition of DDA^+ is accompanied by the oxidation–reduction of mitochondrial NADP(H). Fig. 5 shows the response of NAD(P)H reduced by succinate through the reversed electron transport

pathway. It is seen that addition of DDA^+ cations causes oxidation followed by reduction of nicotinamide nucleotides. Addition of a new portion of DDA^+ cations brings about another oxidation–reduction transition, the subsequent addition of an uncoupler, FCCP, causing strong and irreversible oxidation of NAD(P)H.

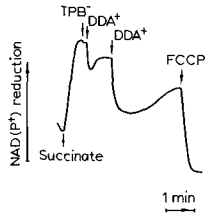


Fig. 5. The effect of DDA^+ cation on the level of $NAD(P)^+$ reduction in beef heart mitochondria. Incubation mixture: 0.25 M sucrose, 0.01 M sodium phosphate (pH 7.5), 1.65 mg/ml mitochondrial protein. Additions: 0.025 M succinate, $1 \cdot 10^{-5}$ M tetraphenyl boron (TPB^-), DDA^+ $1 \cdot 10^{-4}$ M (first addition), $4 \cdot 10^{-4}$ M (second addition), $5 \cdot 10^{-7}$ M FCCP.

DDA^+-induced mitochondrial swelling

It is known that a transient increase in State 4 respiration and cyclic oxidation of NAD(P)H accompany the active transport of Ca^{2+} into the mitochondrial matrix. If a weak penetrating acid (*e.g.* phosphoric acid) is present in the solution, the transport of Ca^{2+} (or K^+) is accompanied by swelling of the matrix, increase in mitochondrial volume and a decrease in light scattering by the mitochondria[4].

Fig. 6 shows the kinetics of mitochondrial swelling measured as a decrease in absorbance at 520 nm. It is seen that incubation of mitochondria in an energized state with DDA^+ and phosphate results in extensive swelling. The effect does not occur if either DDA^+ or phosphate is omitted, or if the energy supply is arrested either by the omission of an oxidizable substrate (or ATP) or by inhibition of the energy-generating system by an uncoupler, a respiratory chain inhibitor (if respiration is used as the energy source) or by oligomycin (in the case of ATP). Among the respiratory chain oxidations the following were found to be able to support DDA^+-

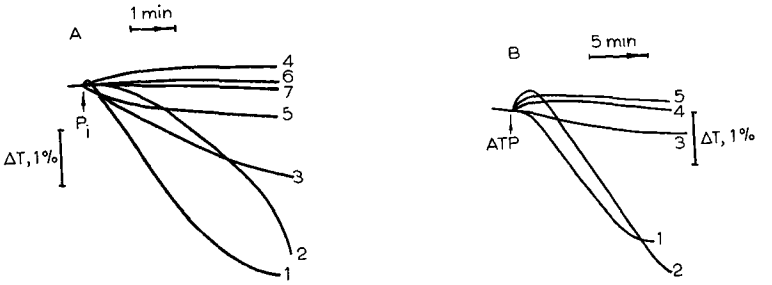


Fig. 6. Kinetics of mitochondrial swelling induced by penetrating ions. Incubation mixture: 0.3 M sucrose, 0.01 M KCl and rat liver mitochondria (1.6 mg of protein/ml). In A all samples were supplemented with 0.01 M succinate; in B with 0.025 M potassium phosphate and $2.5 \cdot 10^{-3}$ M NaCN. Swelling was initiated by the addition of 0.01 M potassium phosphate (A) or $5 \cdot 10^{-3}$ M ATP (B). A. 1, $5 \cdot 10^{-4}$ M $CaCl_2$; 2, $1 \cdot 10^{-3}$ M DDA^+ and $1 \cdot 10^{-5}$ M tetraphenyl boron; 3, $2 \cdot 10^{-7}$ M valinomycin; 4, same as 2 without phosphate; 5, same as 2 without DDA^+; 6, same as 2 *plus* $1.6 \cdot 10^{-6}$ M antimycin; 7, same as 2 *plus* $2.5 \cdot 10^{-5}$ M tetrachlorotrifluoromethyl benzimidazole (TTFB). B. 1, $1 \cdot 10^{-3}$ M $CaCl_2$; 2, $5 \cdot 10^{-3}$ M DDA^+ and $1 \cdot 10^{-5}$ M tetraphenyl boron; 3, as 2 but without DDA^+; 4, as 2 *plus* oligomycin 1 μg/ml; 5, as 2 *plus* $2.5 \cdot 10^{-5}$ M TTFB.

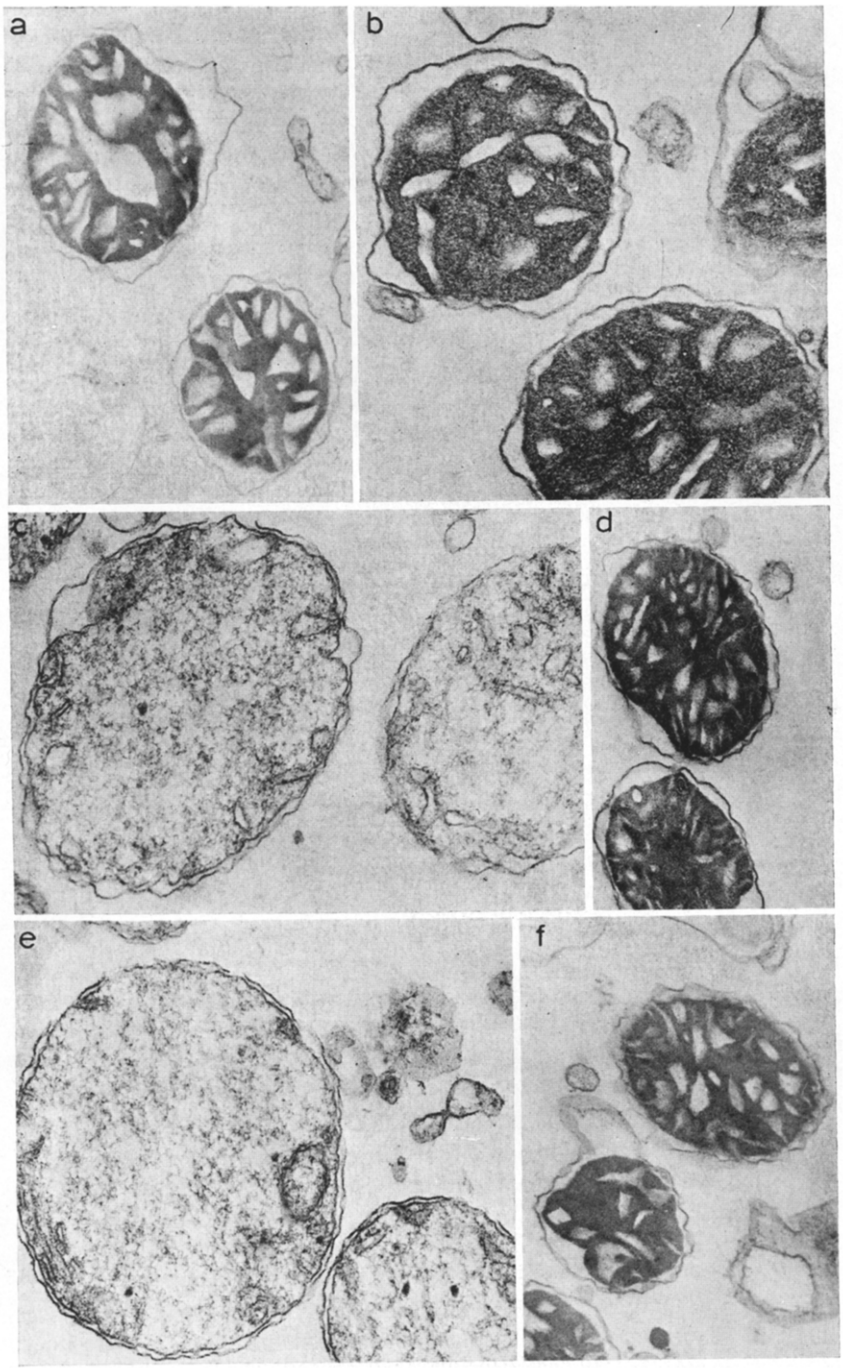


Fig. 7. The effect of penetrating cations on the morphology of rat liver mitochondria. Mitochondria were fixed immediately after measurement of light scattering (see Fig. 6A). Magnification × 34400. a. Control (Fig. 6A, Sample 5). b. Incubation with valinomycin and phosphate (Fig. 6A, Sample 3). c. Incubation with Ca^{2+} and phosphate (Fig. 6A, Sample 1). e. Incubation with DDA^{+} and phosphate (Fig. 6A, Sample 2). d. Incubation with DDA^{+}, phosphate and TTFB (Fig. 6A, Sample 7).f. Incubation with DDA^{+} without phosphate (Fig. 6A, Sample 4).

induced swelling: the oxidation of succinate (or NAD^+-linked substrates) by oxygen or ferricyanide and the oxidation of ascorbate (*plus* *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)) by oxygen. The redox couple, 'NAD^+-linked substrate–fumarate', proved to be ineffective.

Fig. 7 shows electron micrographs of mitochondria prior to and after incubation with penetrating cations and phosphate. It is seen that DDA^+, like Ca^{2+} and K^+ in the presence of valinomycin, increases the matrix space and reduces its density. In the presence of the uncoupler TTFB, or in the absence of phosphate, DDA^+ does not induce any appreciable morphological changes.

It should be mentioned that high doses of DDA^+ (*plus* phosphate) cause irreversible damage to energy-linked mitochondrial functions. This effect resembles the uncoupling after very strong swelling induced by high concentrations of Ca^{2+} and phosphate. In both cases, phosphorylation is inhibited, ATPase and respiration permanently activated and NAD(P)H is irreversibly oxidized. All these changes are probably the consequence of a disturbance of the structural integrity of the mitochondria caused by extensive swelling.

The effects of the synthetic penetrating anions PCB^-, tetraphenyl boron and picrate were studied under identical conditions. These compounds, in concentrations sufficient to affect the energetics of submitochondrial particles (see ref. 1), do not influence the functions of mitochondria. At high anion concentrations some disturbances in energy coupling were apparent. These effects might be due to injury of the mitochondrial membrane upon the incorporation of large amounts of the anions possessing very high affinity for phospholipids.

pH responses of mitochondria to penetrating cations and anions

As seen in Fig. 8A, DDA^+, triphenyl methyl phosphonium and tetrabutyl ammonium added to the mitochondria in State 4, bring about acidification of the incubation mixture. Subsequent addition of FCCP results in alkalinization thus reversing the effect of cations. PCB^-, tetraphenyl boron and picrate anions do not cause any measurable pH changes. Hence, the pH responses of mitochondria proved to be opposite to those of submitochondrial particles. In the latter case, pH changes take place on addition of anions but not cations[1]. Moreover, the directions of pH shifts induced by the addition of cations and anions to mitochondria and particles, respectively, were also opposite. Cations cause an efflux and anions an influx of H^+. An uncoupler, added to mitochondria after a cation, induced alkalinization of the medium; if it was added to the particles after an anion, acidification of the medium was observed (*cf.* Fig. 8A in this and in the previous paper[1]). The ratios of amounts of added cation to extruded H^+ in experiments with mitochondria were usually between 5 and 3 if the initial external concentration of the cation was about $5 \cdot 10^{-5}$ M. Apparently, the significant portion of the added cations remained outside the mitochondria.

pH changes induced by the addition of penetrating cations to mitochondria depend entirely on the operation of an energy supply system. The absence of an oxidizable substrate or of oxygen, as well as inhibition of the respiratory chain, prevents the pH responses caused by cations. The action of antimycin is shown in Fig. 8B. It can be seen that antimycin treatment prior to the addition of DDA^+

prevents acidification of the medium. Antimycin added after DDA^+ reverses the effect of DDA^+ on pH, inducing alkalinization.

Fig. 8B also demonstrates the potentiation of a DDA^+-induced response on the addition of a penetrating anion. It is shown that the rate of acidification of the suspending medium caused by DDA^+ transport is greatly increased by the addition of very small amounts of a penetrating anion ($5 \cdot 10^{-7}$ M tetraphenyl boron in this sample).

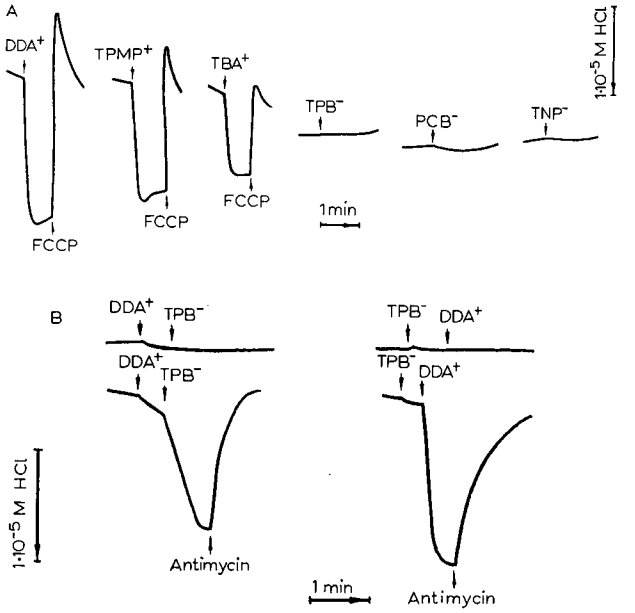


Fig. 8. The kinetics of pH changes on the addition of penetrating ions to rat liver mitochondria. Incubation mixture: 0.25 M sucrose, $1 \cdot 10^{-3}$ M Tris buffer (pH 7.5), $1 \cdot 10^{-3}$ M EDTA, $5 \cdot 10^{-3}$ M $MgCl_2$, $5 \cdot 10^{-4}$ M succinate, $1 \cdot 10^{-6}$ M rotenone, 1.6 g (A) and 1.3 g (B) of protein of rat liver mitochondria per l. Additions: (A) $5 \cdot 10^{-5}$ M DDA^+, triphenyl methyl phosphonium ($TPMP^+$), tetrabutyl ammonium (TBA^+), tetraphenyl boron (TPB^-), PCB^- and trinitrophenol (TNP^-), $1 \cdot 10^{-6}$ M FCCP; the first three samples contained also $1 \cdot 10^{-6}$ M tetraphenyl boron; (B) $1 \cdot 10^{-4}$ M DDA^+, $5 \cdot 10^{-7}$ M tetraphenyl boron, $2 \cdot 10^{-6}$ M antimycin A. In the experiments represented by the top curves of B, antimycin was added before the addition of penetrating ions.

The mechanism for the potentiation of cation permeability by anions is not yet clear. One possible explanation is, for example, that the anions serve as carriers for cations, and it is not the free cation but the neutral anion–cation complex that is transferred across the membrane. The complex would dissociate on the inner side of membrane and the free anion would be transferred to the outer membrane side moving in the electric field. Another possibility would be that anions neutralize some positive charges in the membrane (*e.g.* choline cations of phospholipids) which may hinder the movement of DDA^+ cations across the membrane. If this is the case, it seems possible to explain also the energy-independent (passive) absorption of penetrating anions by mitochondria and submitochondrial particles.

Summarizing the above data, one can conclude that synthetic penetrating ions can be transported in an energy-dependent fashion across the membrane of intact mitochondria, anions being extruded and cations being taken up.

It should be emphasized that the mechanism of energy-dependent transport of synthetic penetrating cations into mitochondria is very similar, if not identical, to that responsible for the energy-linked anion accumulation in submitochondrial particles described previously[1]. In both systems (a) the energy can be provided either by respiration or by ATP hydrolysis ;(b) the utilization of ATP energy (but not of that of respiration) is inhibited by oligomycin; (c) the mechanism of ion transport is nonspecific in regard to the structure of the penetrating compound except for the sign of charge of the ionized atom which is the only factor determining the direction of movement of the ionized molecule; (d) ion transport is coupled with the movement of H^+.

It is of special interest that the ion movements across mitochondrial and particle membranes occur in opposite directions. Electron microscopic evidence suggests that membranes of mitochondria and particles have opposite polarities judging from the position of the 'knobs'[5-7]. Apparently, the opposite orientation of the membrane preconditions the reversed polarity of the electric field that supports ion transfer in mitochondria and particles. The fact that cations are accumulated in mitochondria and anions in 'inside out' sonic particles indicates that mitochondria are positive inside and particles outside, as originally proposed by MITCHELL[8].

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