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DISTRIBUTION OF PERMEANT CATIONS IN RAT LIVER MITOCHONDRIA UNDER STEADY-STATE CONDITIONS

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SUMMARY

The extent of K^+ uptake in aerobic mitochondria is dependent on the valinomycin concentration. Also the extent of uptake of organic cations, such as tetrapropylammonium and tetraethylammonium, is dependent on the tetraphenylboron concentration. The results do not support the hypothesis that permeant cations are distributed in mitochondria in the steady state at electrochemical equilibrium and are in accord with a pump and leak mechanism of ion uptake.

INTRODUCTION

Although the interpretation of ion translocation in mitochondria in terms of electrically driven processes is extensively used[1–8], very few experiments have been reported suitable to test this hypothesis. Tupper and Tedeschi[9,10] have tried to measure the membrane potential in mitochondria with a microelectrode. However, technical reservations have been raised to these measurements[2,11]. The present experiments have been undertaken to test the role of membrane potential on cation distribution in the steady state.

According to the mechanisms implying that the ions move under an electrical force, it is assumed that the permeant cations are distributed across the membrane, under steady-state conditions, at electrochemical equilibrium. It follows that once the permeability of the membrane to K^+ has been established through the addition of valinomycin, it is possible to calculate the dimension of the membrane potential across the membrane from the cation distribution[3–4]. According to other mechanisms, on the other hand, cation uptake is dependent on the interactions of protons and cations with sites in the membrane[12–14]. In this case, the electrochemical potential of the internal cations is higher than that of the external and the cation distribution in the steady state depends on the difference between rates of active transport and of leak. In the present paper we show data which are not compatible with the assumption of the distribution of permeant cations at electrochemical equilibrium.

Abbreviation: EGTA, ethylene glycol bis(β-aminoethylether -*N*,*N*-tetraacetic acid.

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EXPERIMENTAL

Rat liver mitochondria were prepared in 0.25 M sucrose, 5 mM Tris–chloride, 1 mM EDTA, pH 7.4. EDTA was removed in the last washing.

K^+ uptake was measured with a Beckman cationic electrode and a Radiometer pH meter PHM26. H^+ extrusion was measured with a Beckman combination electrode also connected with a Radiometer pH meter. The outputs of the pH meters were recorded on a multi-channel Texas recorder, in order to obtain the simultaneous measurement of the various parameters. The changes in the K^+ concentration in the medium were calculated from titrations carried out at the beginning of each experiment. The absorbance of the mitochondrial suspension was measured at 546 nm, with an Eppendorf photometer, also connected with a recorder. The oxygen uptake was measured simultaneously with a Clark electrode. 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA) was added to prevent Ca^{2+} movement.

RESULTS

Fig. 1 shows the kinetics of a standard experiment. Addition of valinomycin caused a phase of K^+ uptake and of parallel stimulation of respiration. After about 2 min the uptake of K^+ was complete while the rate of respiration became

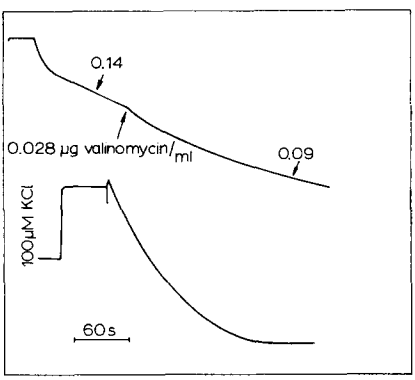


Fig. 1. Respiratory rate and K^+ uptake in the presence of valinomycin. The incubation medium contained 0.2 M sucrose, 10 mM LiCl, 0.5 mM KCl, 2 mM Tris–phosphate, 1 mM β-hydroxybutyrate, 0.1 mM EGTA, pH 7.0. Final volume 2.5 ml. The amount of protein was 5.4 mg/ml. The values in the figure refer to the respiratory rate in μatom per g protein per s.

about equal to that occurring prior to the addition of valinomycin. The rate of the State 4 respiration, following the phase of K^+ uptake, was found to be equal to the initial rate of respiration in the range 0.01–0.3 μg valinomycin per ml and 0.1–0.3 mM KCl. Addition of an uncoupler after the levelling off of the respiration increased the rate of respiration several fold, as expected. It is important here to note that in all the experiments described below, the extent of K^+ uptake was calculated after the mitochondria had reached State 4, where there is no further net movement of K^+ and the mitochondria are assumed to be in a highly energized state.

Fig. 2 shows that the amount of K^+ taken up by rat liver mitochondria incu-

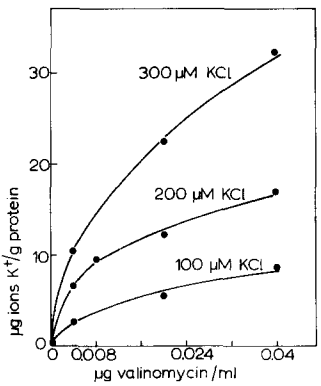
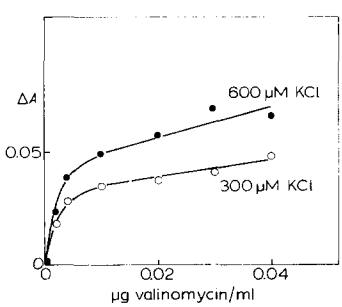


Fig. 2. K^+ uptake at various valinomycin concentrations. The incubation medium was similar to that used in Fig. 1 except that 5 mM LiCl was replaced with 20 mM choline chloride. The amount of protein was 2.7 mg/ml.

bated under aerobic conditions in the presence of low external K^+ concentrations increased with increase in amount of valinomycin added.

Fig. 3 shows that the uptake of K^+, as determined with the K^+ electrode, was paralleled by a decrease of the absorbance at the various valinomycin concentrations. This is due to the swelling of mitochondria, and is expression of the osmotic activity of the translocated species[15].

Bakeeva *et al.*[16] have shown that the rate of active uptake of tetrabutylammonium in liver mitochondria is accelerated by the addition of tetraphenylboron. The molecular mechanism of the tetraphenylboron effect is uncertain and is presently under investigation in our laboratory. In the present work we have used tetrapropylammonium and tetraethylammonium which, at variance from tetrabutylammonium, do not damage the membrane. Both cations do not penetrate the membrane in the absence of tetraphenylboron, while in the presence of tetraphenylboron, their rate of translocation becomes fast.



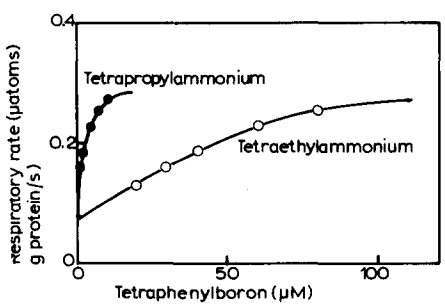


Fig. 3. Absorbance changes accompanying the K^+ uptake. Mitochondria were incubated in same medium used in Fig. 2. The initial absorbance was 1.72 and the amount of protein was 2.7 mg/ml.

Fig. 4. Respiratory rate with organic cations at various tetraphenylboron concentrations. Experimental conditions as in Fig. 1. The amount of protein was 3.8 mg/ml. The respiratory rates were those following the addition of the organic cation.

Fig. 4 shows that the State 3 oxygen uptake rate in the presence of 300 μM tetrapropylammonium and tetraethylammonium increased proportionally to the increase of tetraphenylboron concentrations. With both cations the respiration returned to the level of the initial State 4 rate after the enhancement phase. Similar effects were obtained with picrate instead of tetraphenylboron. Furthermore, the phase of enhanced respiration was accompanied by a decrease of absorbance. The initial absorbance was restored by addition of uncouplers or by ADP. This suggests that the efflux of organic cations is accompanied, as that of K^+, by synthesis of ATP[13].

Fig. 5 shows the extent of aerobic uptake of tetrapropylammonium and tetraethylammonium as measured from the decrease of absorbance of the mitochondria. It is seen that the extent of decrease of absorbance, and therefore of uptake of tetrapropylammonium and tetraethylammonium, is dependent on the concentration of tetraphenylboron or of picrate, in the same way as the extent of K^+ uptake is dependent on the amount of valinomycin.

It has been shown that the rate of K^+ release in dinitrophenol-treated mitochondria increases proportionally to the amount of valinomycin[12]. Fig. 6 shows,

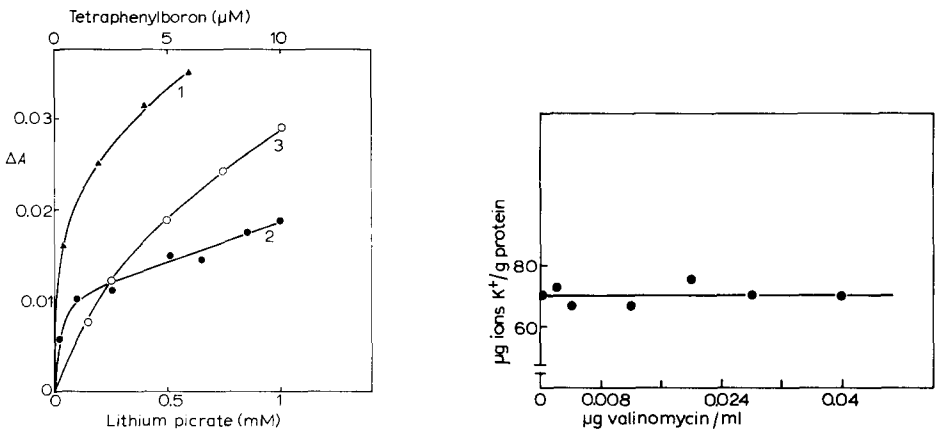


Fig. 5. Absorbance changes during organic cation uptake. Experimental conditions as in Fig. 1. Curve 1: 200 μM tetrapropylammonium and variable concentrations of picrate. Curve 2: 200 μM tetrapropylammonium and variable concentrations of tetraphenylboron. Curve 3: 500 μM tetraethylammonium and variable concentrations of picrate. Initial absorbance was 1.7 and the protein concentration was 2.7 mg/ml.

Fig. 6. Amount of K^+ release at various valinomycin concentrations. The medium contained: 200 μM KCl, 0.2 M sucrose, 20 mM choline chloride, 2.5 mM EGTA, 2.5 mM Tris–chloride, pH 6.8, 50 μM 2,4-dinitrophenol. Final volume 2.5 ml. Amount of protein was 4 mg/ml.

on the other hand, that the extent of K^+ release was independent of the amount of valinomycin added.

Fig. 7 summarizes the relationship between extent of aerobic K^+ uptake and relative rates of aerobic K^+ uptake and of rotenone-induced K^+ efflux at various valinomycin concentrations. The rate of rotenone-induced K^+ efflux: (a) was faster than the rate of aerobic K^+ uptake at low valinomycin concentrations and (b) it ranged from 0.2 to 0.7 μg ions per g protein per s at low and high valinomycin concentrations, respectively. The rates of K^+ efflux may be taken as expression of the leak of the anaerobic membrane (in respect to energy dissipation or more

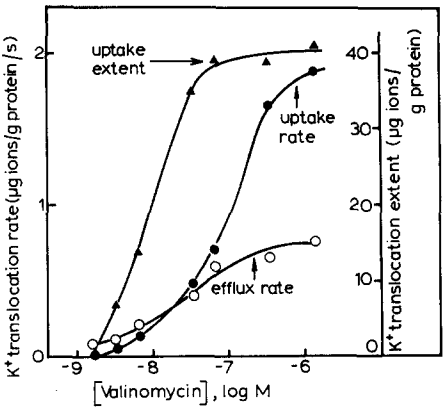


Fig. 7. Relationship between rates of K^+ translocation under aerobic and anaerobic conditions and extent of K^+ uptake. The medium contained: 0.2 M sucrose, 20 mM choline, chloride, 2 mM Tris–phosphate, pH 6.8, 1 mM β-hydroxybutyrate and 100 µM KCl. Amount of protein was 4.8 mg/ml. The anaerobic rate was that observed after the addition of rotenone at the end of the phase of aerobic uptake.

specifically to H^+). That the rate of anaerobic efflux is faster than that of the aerobic uptake is understandable if one assumes that while the former indicates only the passive leak, the latter is the sum of two processes, an active uptake and a passive leak (*cf.* below in Discussion).

DISCUSSION

Valinomycin is known to increase the permeability of natural and artificial membranes to K^+ and its addition to mitochondria results in K^+ uptake, H^+ extrusion, swelling and stimulation of the respiration[17]. The rates of all these processes depend on the amount of valinomycin added, there being a roughly linear relationship between rates of K^+ and of O_2 uptake on one side and concentration of valinomycin on the other[17,18]. This is to be expected since the change in metabolic state depends on the energy demand put by the ion translocation and this is a function of the increase of permeability induced by valinomycin. However, the question arises as to the difference of electrochemical potential of the K^+ across the valinomycin-treated membrane. In the mechanisms assuming an electrically driven translocation[1–3,5–7] the $[K^+]_i/[K^+]_o$ ratio in the steady state, where the membrane potential is constant and maximal, should be independent of the extent of valinomycin-induced increase of permeability if the $[K^+]_o$ and $[H^+]_o$ are constant. Therefore, the amount of K^+ uptake should also be independent of the concentration of valinomycin. Instead, the present findings show a valinomycin dependence of the $[K^+]_i/[K^+]_o$ ratio in the steady state. Three *ad hoc* assumptions can be made to account for these observations within the hypothesis of a membrane potential: (a) The mitochondrial membrane possesses, as supposed by Mitchell[1], a specific H^+/K^+ antiporter which catalyzes the transport of K^+. The operation of the antiporter would reduce the amount of K^+ uptake especially at low valinomycin concentration. (b) Part of the K^+ taken up might be bound. This would result in a constant $[K^+]_i/[K^+]_o$ ratio, even if the amount of K^+ taken up is variable. (c) The

variation of the valinomycin concentration influences some parameter of the transport mechanism (extent of K^+ dissolved in the membrane, surface potentials) not primarily related with the active translocation and the cation distribution.

However, all three assumptions are unlikely for the following reasons. First, the proposal of an H^+/K^+ antiporter may hold for the uptake of K^+ but not for that of the organic cations since one of the properties of the K^+ antiporter is its specificity. Second, Fig. 3 indicates that the translocated K^+ is osmotically active at all valinomycin concentrations. Third, Fig. 6 indicates that the extent of K^+ release, which conforms to the same transport parameters in a roughly symmetrical membrane, is independent of the amount of valinomycin.

On the other hand, the data reported here are compatible with the mechanism of the electroneutral proton pump[12–14]. In this scheme the cations are not at electrochemical equilibrium and the extent of uptake depends, under conditions of adequate energy supply on (a) the equilibrium constant of the exchange reaction and (b) the presence of a cation leak in the membrane. Evidence for such a leak has been provided by Harris *et al.*[19] who used measurements with $^{42}K^+$. Other evidence for a leak is provided by the experiment reported in Fig. 7, where the rates of aerobic K^+ uptake and of anaerobic K^+ efflux were compared at various valinomycin concentrations. The marked dependence of the extent of aerobic uptake on the amount of valinomycin supports a mechanism where a nearly constant leak rate balances a slow uptake rate at low valinomycin concentrations.

It may be argued that the smaller K^+ uptake at low valinomycin concentrations be due to an H^+ leak. Such a leak would decrease the membrane potential as well as the $[K^+]_i/[K^+]_0$ ratio. It should be recalled, however, that in the electrophoretic mechanisms (electrogenic proton pumps), although the magnitude of the membrane potential is dependent on the H^+ leak, the magnitude of the H^+ leak is independent of the valinomycin concentration. Therefore, also the membrane potential is independent of the valinomycin concentration. That the low respiratory rate be due not to State 4 but to inhibition of respiration is in contrast with the subsequent respiratory stimulation by uncouplers. Incidentally, respiratory inhibition would have required a large amplitude swelling with loss of nucleotides.

The present experiments, which are not compatible with a distribution of cations at electrochemical equilibrium, may be taken as an evidence against the occurrence of a large membrane potential in intact mitochondria. This evidence adds to that already reported on the kinetics of the cation translocation[12] and the dependence of the $K^+/\sim$ ratio on the external and internal H^+ and K^+ concentrations[13].

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