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The Mechanism of Energy-Dependent Ion Transport in Mitochondria

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Summary. The transport of potassium, sodium and various anions in rat-liver mitochondria was studied mainly by analysis of ion content and water compartmentation of the mitochondrial pellet. A comparison of spontaneous transport with valinomycin- or gramicidin-stimulated transport is made. The rate or extent of uptake, the internal concentrations and the concentration ratio (C_{in}/C_{out}) are calculated and compared to test existing models for ion transport in mitochondria.

Several models of ion transport in mitochondria are based on a cation-pump which is directed inward. This hypothesis is rejected because of the following findings: (1) Valinomycin stimulates the rate of potassium uptake but does not increase the potassium concentration ratio that can be actively maintained in a steady state (in which there is no potassium flow). (2) Valinomycin greatly stimulates the efflux of ^{42}K from mitochondria during the process of potassium accumulation. When potassium accumulation is stimulated the flux ratio, i.e. influx/efflux, decreases; in the presence of valinomycin this ratio approaches 1. (3) In the presence of gramicidin, the concentration ratios of potassium and sodium are about the same under a variety of conditions. These findings indicate that potassium and sodium transport are passive processes of relaxation towards electro-chemical equilibrium (of the potassium and sodium). In high external potassium concentrations the extent of potassium uptake is limited by the permeation of anions; of the permeating anions multivalent acids support a higher extent than monovalent acids. It was found that succinate, acetate and oxalate which are transported together with potassium are distributed in accordance with the ΔpH and without any relation to the potassium concentration ratio. These findings are compatible with the hypothesis that an outward-directed proton pump creates an electrical potential gradient, which shifts the equilibrium state for the cations and drives sodium and potassium inward, and also creates proton gradient that is the driving force for anion transport.

Numerous studies of ion transport in mitochondria during the last decade revealed that when energy is supplied, either in the form of reducing substrate or as ATP, various ions are transported across the mitochondrial

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membranes (Lehninger, Carafoli & Rossi, 1967). Divalent cations such as Ca^{++}, Mg^{++} and Mn^{++} can be accumulated rapidly and reach high internal content. The spontaneous accumulation of potassium is slow and limited (Rottenberg & Solomon, 1965), but can be stimulated by valinomycin or similar compounds (Pressman, 1965). Sodium is not taken up unless stimulated by gramicidin or similar compounds (Chappell & Crofts, 1965; Pressman, 1965). Many anions can be accumulated as well, in particular metabolic intermediates (Klingenberg, 1970). One striking exception to the energy-driven accumulation of ions is the proton transport which is directed outward (Mitchell, 1968).

All these processes are often referred to as active transport because of their strong dependence on metabolic energy. Several carriers for anions have been postulated (Mitchell, 1968; Klingenberg, 1970) and the existence of Ca^{++} carrier has been suggested as well (Reynafarje & Lehninger, 1969). However, these criteria alone are not sufficient to establish direct coupling to metabolism since the dependence on metabolic energy can be mediated via gradients of other ions, electrical potential, etc. Indeed, most of the proposed models do not assume a specific "pump" for each of the transported species but postulate a single, more or less specific pump, actively maintaining gradients, while some other transport processes derive their energy from these gradients. Although the proposed models differ in many details they can be classified into three general classes.

I. Inward-Directed Electrogenic Cation-Pump (Fig. 1a)

In this model, the cation(s) is pumped inward, which results in an electrical potential that is positive inside; this potential induces proton ejection and/or anion uptake (which are both "passive" movements since their only driving force is their electrochemical potential gradient). Since there are several cations that can be transported inward, one may postulate either several specific pumps or one nonspecific pump in which the specificity can be modified by various compounds such as valinomycin and gramicidin (Cockrell, Harris & Pressman, 1966; Harris & Pressman, 1969).

II. Outward-Directed Electrogenic Proton Pump (Fig. 1b)

In this model, which in some of its aspects is symmetric to Model I, it is postulated that protons are ejected by the pump while the cation moves in down an electrochemical gradient (Mitchell, 1968). The uptake of anions must be explained here as being coupled to another ion flow (since the

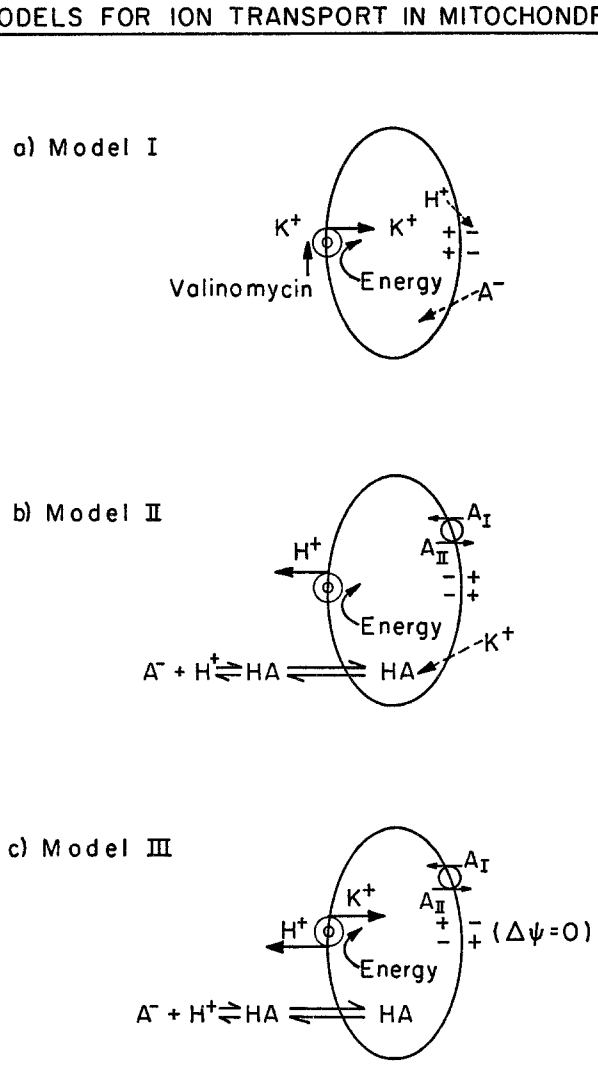

Fig. 1. Models for ion transport in mitochondria. (*See* details in text)

potential which is negative inside would cause ejection of permeable anions). It is normally assumed that most anions are not permeant as such but either exchange for hydroxyl, or exchange with other anions via special exchange systems, or penetrate in co-transport with protons in the form of acids (undissociated). In either case, energy for anion uptake is derived from the proton concentration gradient which is maintained by the proton pump. At steady state, according to this model,

$$A^-_{in}/A^-_{out}=H^+_{out}/H^+_{in}=\sqrt{A^{-2}_{in}/A^{-2}_{out}}.$$

Thus, in this model the metabolic energy is converted by the electrogenic proton pump into a proton concentration gradient and an electrical potential gradient, whose relative magnitude depends on the membrane permeability. The electrical potential gradient is the driving force for cation uptake which moves passively down an electrochemical gradient while anion uptake is driven by the proton concentration gradient either by direct coupling or by exchange with other anions that are directly coupled to proton (or hydroxy) flow.

III. A Neutral Cation-Proton Pump (Fig. 1c)

In this model, the cation-proton exchange does not result in a membrane potential. Both cation uptake and proton ejection are directly coupled to metabolism. The anion transport associated with this model should be of the same type as in Model II (Massari & Azzone, 1970).

In addition, it is possible to suggest that anion pumps exist as well, or that the same pump can transport cations as well as anions (Pressman, 1970).

This study will examine the predictions of the various models as related to potassium, sodium and anion transport in rat-liver mitochondria. Both Models I and III postulate active inward cation pumping in contrast to Model II. Several criteria can be applied to test this hypothesis. The difficulty is that almost all require the determination of both the internal concentration of the cations and the membrane potential. The membrane potential can be computed within the assumptions of the different models. Model I predicts a potential positive inside that can be computed from the distribution of permeant anions (but not from the accumulated cations since they are presumably not in equilibrium) (Harris & Pressman, 1969); Model II predicts a potential negative inside that can be computed from the permeant cation distribution (Mitchell & Moyle, 1969*a*; Rottenberg, 1970) (but not from the "permeant" anions since they are presumably coupled to proton transport and are not in equilibrium); while Model III predicts that there is not any significant membrane potential, and in any case neither the cations nor the anions are in electrochemical equilibrium. An important consequence of an ion pump is the existence of a metabolism-dependent "static head"; that is, a state in which metabolic energy is expended to maintain a maximal electrochemical potential gradient of the actively transported ion (Kedem & Caplan, 1965). Any factor that is supposed to stimulate pumping should increase the potential of the static head; thus, comparison of the static heads of various ions under various conditions might enable us to distinguish between passively distributed ions and those that are actively pumped. Another criterion frequently employed is the flux ratio of the

transported ion (Ussing, 1949). Transport processes driven by a pump exhibit flux ratios that deviate strongly from those expected from the electrochemical potential gradient. Again, any stimulation of pumping should result in further deviation caused by the increase in the active flux. Finally, a pump should show all the characteristics of a carrier mechanism such as saturation kinetics specificity toward ions and/or competitive inhibition between them.

Materials and Methods

Rat-liver mitochondria are prepared and washed once in 0.25 M sucrose as previously described (Rottenberg & Solomon, 1966). The analysis of the pellet water is done gravimetrically, sodium and potassium content by flame photometry. Water compartmentation is determined by ^{131}I-serum albumin and ^{14}C-sucrose as previously described (Rottenberg & Solomon, 1969). Other ^{14}C-labeled compounds were determined by the same procedure. ΔpH was determined by the use of ^{14}C-DMO (Addanki, Cahill & Sotos, 1968) using the sucrose space as correction for the internal space.

^{42}K was counted in a Nuclear Chicago counter (model 186). Filtration was done through a double layer of Millipore filter (3.0 and 0.8 μ). Protein was determined by the biuret method (Layne, 1957). Valinomycin was a gift from Dr. L. C. MacDonald of the National Research Council, Saskatoon, Canada; gramicidin was from Calbiochem. All reagents were of analytical grade.

Results

The Potassium Concentration in the Inner Compartment of Mitochondria

The pellets of freshly prepared rat-liver mitochondria, which were isolated in sucrose medium (0.25 M) without any addition of salts, did not contain any significant amount of sodium (less than 1 nmole/mg dry wt). In contrast, the pellet retained up to 100 nmoles/mg dry wt potassium even after repeated washing with sucrose, provided that the washings were done in the cold (4 °C). When incubated at 30 °C in the sucrose medium, they gradually lost most of their potassium. After 30 min of incubation they retained only about 5 nmoles/mg dry wt. This amount is retained even on prolonged incubation and repeated washing with sucrose medium which contained less than 10^{-5} M potassium. Thus, it seems that this amount of potassium binds strongly to the mitochondria. Incubating mitochondria at 30 °C in sucrose medium that contains KCl resulted in loss of potassium as long as the external concentration was less than 100 mM, but uptake was observed in concentrations higher than 100 mM. In these experiments, the total osmolality was kept constant at 250 mosm. The uptake of potassium by the mitochondria at these concentrations indicates that the initial potassium concentration in the mitochondria is about 100 mM. Fig. 2 shows the

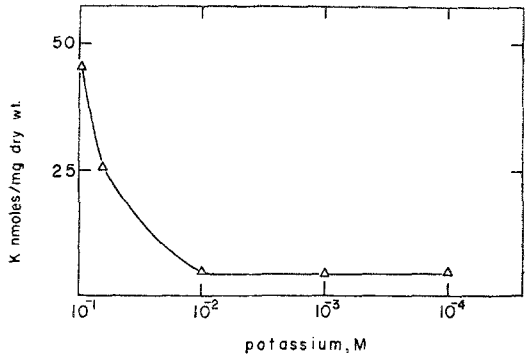

Fig. 2. The effect of external potassium concentration on potassium binding by mitochondria. Mitochondria were incubated in a medium that contained different KCl concentrations and sucrose in concentrations that brought the total osmolality to 250 mosm. Temp = 30 °C. Bound potassium was calculated from the potassium and water content after 30 min of incubation

amount of bound potassium as a function of external potassium concentration. This amount is calculated assuming that the concentration in the pellet after a 30-min incubation at 30 °C is identical with the concentration in the medium. Thus, from the measured values of the water content and the potassium content of the pellet and the known potassium concentration of the medium, a value for the amount of potassium which is not in solution, i.e. bound potassium, is obtained. It is observed that in addition to the strongly bound potassium (5 nmoles/mg dry wt) there is a weak binding which is concentration-dependent and can amount to 45 nmoles/mg dry wt in 10^{-1} M potassium.

To determine the internal concentration of potassium we incubated the mitochondria in the presence of 10^{-3} M EDTA and 10^{-3} M ATP. Under these conditions the mitochondria retain most of their potassium and adjust their volume quickly in response to a change in medium osmolality. The subsequent passive gain or loss of potassium presumably depends on the resulting internal concentration caused by the osmotic adjustment of volume.

Fig. 3 shows the effect of the medium osmolality on potassium movements. The external potassium concentration is 75 mM while the osmolality is varied (by sucrose) from 150 mosm to 350 mosm. It is observed that when the osmolality is 150 mosm there is a gain of potassium. At 200 mosm there is no change, while in higher osmolality there is a loss. Thus, it seems that at 200 mosm the external potassium concentration equals the internal concentration; that is, 75 mM. From this result one can estimate the internal concentration of freshly prepared mitochondria at any other osmolality.

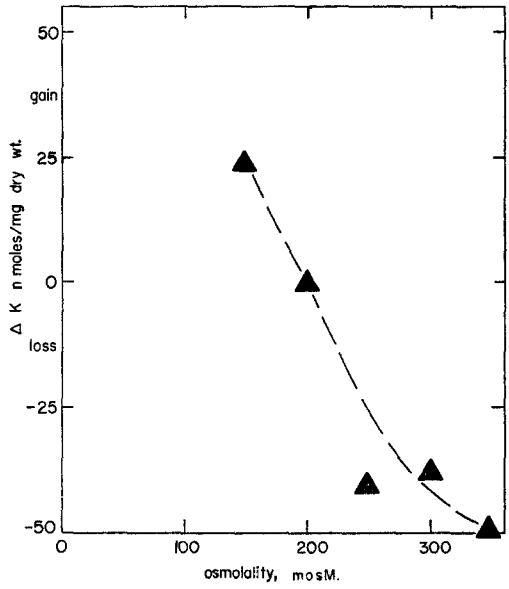

Fig. 3. The effect of medium osmolality on passive potassium transport. Mitochondria were incubated in a medium of 75 mM KCl, 1 mM sodium ATP and 1 mM EDTA and sucrose at various concentrations to obtain the indicated total osmolality. The pellet was analyzed after 20 min of incubation. Temp = 30 °C, pH 7.0

Table 1. Analysis of pellet content and compartmentation at 250 mosm[a]

A. *Water content*	μliters/mg dry wt	
Total water content	2.38	
Sucrose permeable space	1.55	
Albumin permeable space	0.69	
Outer compartment (sucrose space − albumin space)	0.86	
Inner compartment (total water − sucrose space)	0.83	
B. *Potassium content and concentrations*	nmoles K/mg dry wt	mM K
Pellet content	145	
Medium concentration		10
Apparent inner concentration (not corrected for binding)		157
Inner concentration (after correction for binding)		98

[a] The medium contained: 20 mM Tris-HCl, pH 6.9; 5 mM Na_2 succinate; 2.25 mM Na_2 ATP; 10 mM KCl; 1 μg/ml oligomycin; 1 mM NaCN, and sucrose to complete osmolarity to 250 mosm.

At 250 mosm the internal concentration should be about 95 mM. (The results of the previous experiment showed that it must be about 100 mM.)

Table 1 shows the analysis of mitochondrial pellets incubated in the cold in a medium of low potassium. Both the water content and compartmentation and the ion content are determined from the same sample. Part A shows the compartmentation at 250 mosm as determined from the ^{14}C-sucrose and ^{131}I-albumin spaces. These data are used in part B to calculate the concentration of the potassium in the inner compartment by assuming that both the extramitochondrial water and outer compartment, i.e., the sucrose space, have the same potassium concentration as the supernatant. The calculated concentration of the potassium in the inner compartment without correction for binding is 157 mM. When corrected for the weak binding (assuming that the binding is dependent on the concentration of potassium inside the mitochondria and using values of Fig. 2) the corrected estimated concentration is 98 mM. This estimate, arrived at independently from the two preceding estimates, gives a reasonable value of 10^{-1} M as internal concentration at this osmolality.

Comparison of Valinomycin-Stimulated and Spontaneously Energy-Dependent Potassium Uptake

Mitochondria can accumulate potassium even at fairly low potassium concentration in the medium provided that an energy source and permeant anions are present (Rottenberg & Solomon, 1966). This process can be stimulated by valinomycin (Pressman, 1965). During potassium accumulation the mitochondria swell in an isotonic process and therefore the internal concentration which is initially high increases only slightly (Rottenberg & Solomon, 1969). The stimulation of the uptake by valinomycin must be explained in light of the extensive evidence that valinomycin increases the membrane permeability to potassium (*see*, for instance, Chappell & Haarhoff, 1967). If the electrochemical potential of potassium inside the mitochondria is higher than outside as implied by Models I and III then one would expect that valinomycin would increase the leak of potassium and decrease the rate and extent of uptake. To explain the stimulation proponents of Models I and III must assume a specific interaction between valinomycin and a pump, while a barrier exists to potassium permeability which is not affected by valinomycin (Pressman, 1970). Thus, the stimulating effect of potassium is interpreted as a stimulation of the pump alone without effect on the leak pathway. Model II explains the stimulation by postulating that the electrochemical potential of potassium is higher *outside* (caused by the proton pump electrogenicity) and the increased permeability caused by the valinomycin causes a faster inward movement. In this model, when net uptake

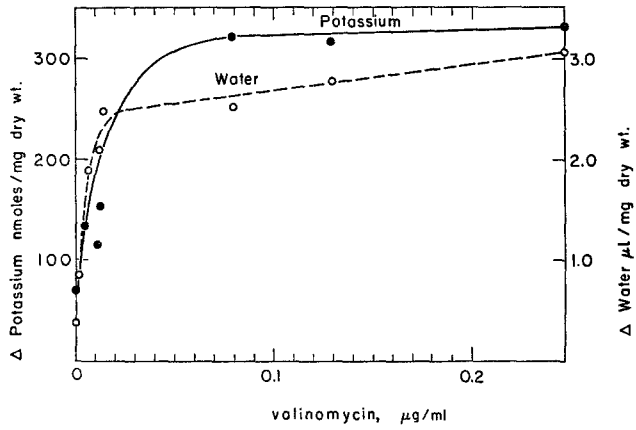

Fig. 4. The effect of valinomycin concentration on potassium and water uptake. Mitochondria were incubated in 10 mM KCl, 5 mM sodium succinate, 2.5 mM sodium ATP and 210 mM sucrose. A mixture of 95% O_2 − 5% CO_2 was passed over the suspension which was vigorously shaken. pH 6.5, Temp = 30 °C. Incubation time 10 min

vanishes, equilibrium is established in contrast to the other models that consider this a static head. Fig. 4 shows the dependence of potassium uptake and water uptake on the valinomycin concentration. It should be pointed out in this connection that the kinetics of potassium uptake is bi-phasic: a rapid uptake with a half time of 10 to 30 sec and a slow one with a half time of 5 to 10 min (Rottenberg, 1968). Our results are related to this latter phase. Most studies of potassium transport which are done with the aid of a glass electrode deal with the rate of the initial rapid uptake and the extent which is given is actually the amount taken during the rapid phase. In several cases, particularly when phosphate is the permeant anion, the fast initial uptake is followed by loss. However, comparison of the uptake as determined by pellet analysis with simultaneous cationic electrode determination shows that there is very good agreement between the two methods (Rottenberg, 1968). Fig. 4 is a simple saturation curve which is compatible with Model II. With Models I and III, it is hard to see why an increase in valinomycin after saturating the pump would not cause its usual effect, that of increasing leak and thus diminishing the rate of uptake in higher concentrations.

Fig. 5 shows the effect of the external potassium concentration on the rate of uptake in the presence and absence of valinomycin. Under these conditions the rate is a linear function of the concentration over the whole range. The fact that the rate dependence on valinomycin concentration shows a typical saturation curve, whereas the dependence on potassium (with high valinomycin) does not, is compatible with Model II which postulates that

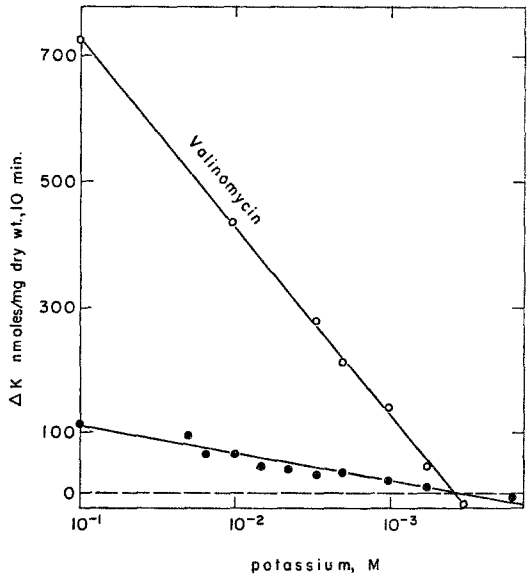

Fig. 5. The effect of potassium concentration on the rate of potassium uptake. Conditions are as in Fig. 4, except for the potassium concentration. The valinomycin concentration, when present, is 0.15 μg/ml

in the presence of excess valinomycin the membrane becomes essentially permeable to potassium.

A more important aspect of Fig. 5 is that while it is evident that valinomycin greatly accelerates potassium transport, both curves cross the zero line at the same point. This point was further checked by the use of cationic glass electrodes. Although the exact external potassium concentrations in which the uptake vanishes vary from 0.2 mM up to 0.8 mM, depending on the mitochondrial preparation, medium composition, and in particular osmolarity, it was not significantly altered by valinomycin provided that the substrate concentration was high. Moreover, in high potassium concentrations where the transport rate and extent is affected by valinomycin the final potassium concentration gradient which is reached at steady state (i.e., when potassium uptake vanishes) is not affected by valinomycin (*see* Rottenberg & Solomon, 1969).

This indicates that the equilibrium or "static head" state is the same with or without valinomycin. Models I and III assume this "static head" to represent the potential of the pump to hold a gradient. However, if the valinomycin effect is to stimulate pumping one would expect that with valinomycin the static head potential would be higher than without it. In contrast, Model II predicts that there would be no difference since in this

state the potassium is at equilibrium which should not be affected by valinomycin (only the relaxation towards equilibrium is accelerated by valinomycin, but the equilibrium itself is not changed[1]). If one accepts the interpretation of Model II, it is possible to calculate the membrane potential from the equilibrium values.

Since

$$\Delta\tilde{\mu}\mathrm{K} = RT\ln \mathrm{K_{in}}/\mathrm{K_{out}} + F\Delta\psi = 0$$

$$\Delta\psi = (-RT/F)\ln(\mathrm{K_{in}}/\mathrm{K_{out}}).$$

Utilizing the results of Fig. 5 and the estimated internal potassium concentration, we obtain a membrane potential of about 130 mV. Mitchell and Moyle (1969*a*) have estimated the mitochondrial membrane potential by a similar method and obtain much higher values (up to 200 mV). These values were obtained in much lower external concentrations in which the internal potassium content is very low and should be corrected for binding (*see* Fig. 2 and Table 1). At external concentrations which are comparable to ours [their Table 1 (iii)], they got a value of 139 mV which is comparable to our value.

Flux Ratio During Net Potassium Uptake

Mitochondria were incubated in the cold for 5 hr in the presence of 20 mM KCl and ^{42}K in sucrose medium (0.25 M). At the end of the incubation, the specific activity of the pellet was equal to that of the medium thus indicating that all the potassium was exchanged. The ^{42}K-loaded mitochondria were collected by centrifugation and then diluted in cold medium; the efflux of ^{42}K was followed either by centrifugation or by filtration through millipore filters. The specific activity of both the medium and the mitochondrial pellet was followed. Fig. 6 shows that in the cold the efflux was very slow, and that raising the temperature to 30 °C accelerated the efflux considerably.

In these experiments, the net potassium transport was also determined (Table 2). While net uptake is increased as well at the high temperature, the effect on efflux is much more pronounced. Fig. 7 shows the effect of the addition of valinomycin. The efflux is so rapid that immediately after addition the specific activity reaches its minimal value which is identical with the medium specific activity. The subsequent increase in the pellet ^{42}K content is simply a reflection of the increase in potassium content as seen in the figure, while the specific activity does not change. This means that the fluxes

1 This is true only in low potassium concentration in which the valinomycin does not affect the membrane potential.

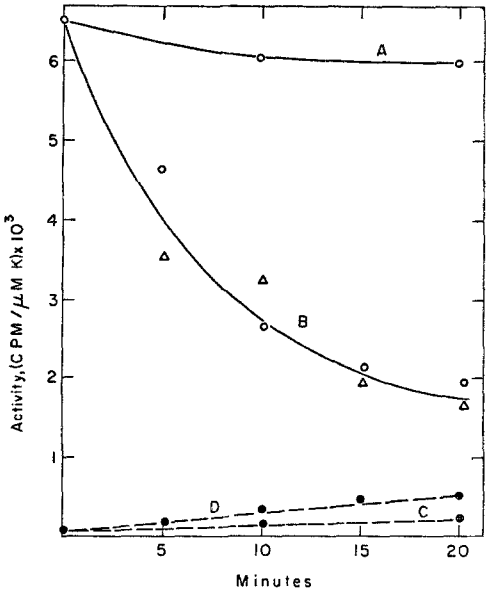

Fig. 6. ^{42}K efflux as a function of temperature. Incubation medium is the same as in Fig. 4. Mitochondria were equilibrated with ^{42}K for 5 hr at 4 °C in a medium of 20 mM KCl and 0.25 M sucrose. Curve *A* shows the pellet specific activity (cpm/μM potassium) in the cold (4 °C). Curve *B* shows the pellet specific activity at 30 °C (circles are from centrifugation analysis and triangles from filtration through Millipore filters). Curve *C* shows the supernatant specific activity in 4 °C and curve *D* shows the supernatant specific activity in 30 °C

Table 2. Potassium flux ratio during the initial periods of net uptake[a]

Condition	Fluxes (nmoles/mg dry wt/min)			Flux ratio
	Net	Efflux	Influx	Influx/Efflux
Standard medium, 4 °C	0.7	0.07	0.77	11.0
Standard medium, 30 °C	7.1	8.1	15.2	1.89
+Valinomycin, 23 °C	41.0	1,000.0[b]	1,041.0	1.04

[a] The results are calculated from experiments of the type shown in Fig. 6 and from Fig. 7 as explained in the text.

[b] This is a minimal value estimated as follows: since all the potassium exchanges within 20 sec after the addition of valinomycin, the half time must be smaller than 5 sec, which requires that the efflux would be >1,000 nmoles/mg dry wt per min.

in the presence of valinomycin are so fast that it is only possible to estimate their lowest possible value.

From experiments such as those of Fig. 6 the individual fluxes and a flux ratio are calculated. The rate of efflux is estimated from the loss of ^{42}K

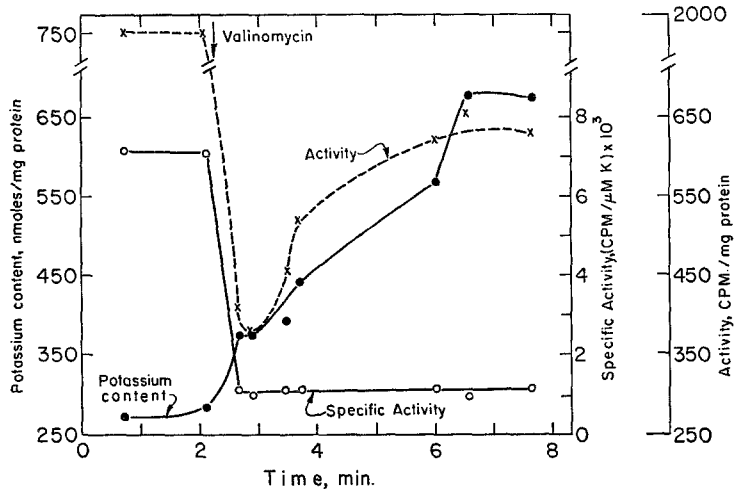

Fig. 7. ^{42}K efflux and net potassium uptake after addition of valinomycin. Procedure as in Fig. 5 except that all the data are from filtration through Millipore filters. Temp = 24 °C. Valinomycin 0.15 μg/ml. The scales on the right show the pellet activity (cpm/mg, ---x---) and the pellet specific activity (cpm/μM potassium, —o—). The scale on the left shows the potassium content (mM/mg protein, —•—)

from the pellet during a 5-min interval and the average specific activity during that time. Only the initial data are used because later a significant increase in ^{42}K specific activity of the medium occurs. The influxes are calculated from the difference between the rate of net transport and the efflux. Table 2 shows the results of these calculations. Since it was not possible to measure the efflux in the presence of valinomycin but only to estimate its lower limit, the flux ratio estimated for this system represents its upper limit.

It is observed that increasing net transport either by increasing temperature or by valinomycin treatment results in decreased flux ratio. Indeed in the presence of valinomycin the flux ratio is so close to 1 (and this is its upper limit) that the system can be considered to be practically at equilibrium. As discussed in the introduction, stimulation of a potassium pump should result in an increased flux ratio but here indeed the stimulation of net uptake is correlated with a decreased flux ratio. This observation is not compatible with the prediction of Models I and III but is predicted by Model II, in which potassium transport stimulation is considered to be caused by a faster relaxation towards equilibrium.

Effect of Gramicidin on Sodium and Potassium Transport

Gramicidin was found to affect both sodium and potassium transport in mitochondria (Chappell & Crofts, 1965; Pressman, 1965). In other

Table 3. Dependence of the concentration ratio of sodium and potassium in the presence of gramicidin on medium composition[a]

No. of exp.	External K (mM)	External Na (mM)	ΔK nmoles / mg dry wt	ΔNa nmoles / mg dry wt	K_{in}/K_{out}	Na_{in}/Na_{out}
2	6	6	0	+72	4.0	3.9
12	20	20	+70	+120	3.4	3.3
2	10	20	+50	+150	2.3	2.2
2	40	20	+100	+105	2.1	2.0
2	40	10	+105	+55	1.8	1.7
2	30	25	+80	+200	2.5	2.4

[a] Conditions are the same as in Fig. 4 except for the sodium and potassium concentrations as indicated; gramicidin concentration is 17 ng/ml. Incubation time is 20 min.

natural membrane systems as well as in black lipid membranes it was found to increase the permeability to most univalent cations including protons (Chappell & Haarhoff, 1967). The effect on mitochondria is very complicated since the magnitude and direction of ion movement are strongly dependent not only on the gramicidin concentration but also on incubation conditions such as sodium and potassium concentration.

At optimal gramicidin concentration (17 ng/ml) there is potassium uptake when external concentration is higher than 5 mM but potassium is lost if the concentration is lower. When sodium is present there is always sodium uptake but this uptake decreases when there is massive potassium uptake.

These complicated effects can be understood if the concentration ratios of sodium and potassium are calculated and compared. Table 3 shows the net transport and concentration ratios of sodium and potassium that were found in different cation concentrations. It is observed that in each case the concentration ratio of potassium is almost indentical to that of the sodium (potassium is slightly higher and this is probably caused by the potassium binding, which is not adjusted in these calculations). Thus, if we assume that the system approaches the steady state with equal concentration gradients for sodium and potassium we can see why in certain cases potassium leaks out while sodium is taken in, whereas in other conditions increased potassium uptake is associated with a decreased sodium uptake. The fact that in any case the same concentration gradient for sodium and potassium is established is not easily explained by Models I and III. In Table 3 we see that increasing the external ion concentration from 6 to 20 mM results in decreased concentration ratios. Thus, if a cationic pump maintains this

gradient, this concentration range must be well into the saturation region since increasing the external concentration seems to decrease the gradiant that can be held. Since at equal concentrations equal gradients are maintained, the pump if it exists, must be nonspecific and therefore changing the external concentration ratio of sodium and potassium should, by competitive inhibition, change the rate of pumping and allow the maintenance of a higher concentration gradient for the ion with higher external concentration. But Table 3 shows that regardless of the relation between the external sodium and potassium, the concentration gradient is the same. Model II of course predicts this behavior; since both sodium and potassium should be in equilibrium and since both "see" the same membrane potential, the concentration gradients must be the same. Thus, a membrane potential that is computed in this system from the sodium concentration gradient is the same as that which is computed from the potassium gradient. These findings are compatible with the hypothesis that gramicidin brings both sodium and potassium into electrochemical equilibrium.

The fact that increasing the gramicidin concentration reduced the cation concentration gradient, which is interpreted as a reduction of membrane potential in Model II, is explained as a result of increased proton permeability (Chappell & Haarhoff, 1967). But the decrease of the gradient by high ionic concentration in constant gramicidin concentration (Table 3) is not easily understood. This effect which is not well correlated with external ion concentration seems to show excellent correlation with the pellet water content. Fig. 8 shows the relation between water content and the sodium concentration gradient from various experiments, in a variety of conditions, but in the same gramicidin concentration.

It shows that when the water content increases from 3 to 4 μliters/mg dry wt, a sharp decrease in Na_{in}/Na_{out} occurs (normal water content is about 2.5 μliters/mg dry wt, *cf.* Table 1). Thus, it is possible that swelling, at least in the presence of gramicidin, causes increased permeability to protons and therefore a decrease in membrane potential. The membrane potential found in the presence of valinomycin and high concentration of potassium is similarly low when extensive swelling occurs (Rottenberg, 1970).

Effect of Anions on Energy-Dependent Potassium Uptake

It is well known that the extent of potassium uptake in rat-liver mitochondria is dependent on the presence of anions in the incubation medium (Cockrell *et al.*, 1966). Since it is reasonable to assume that this dependence reflects the co-transport of this anion together with the potassium, it is

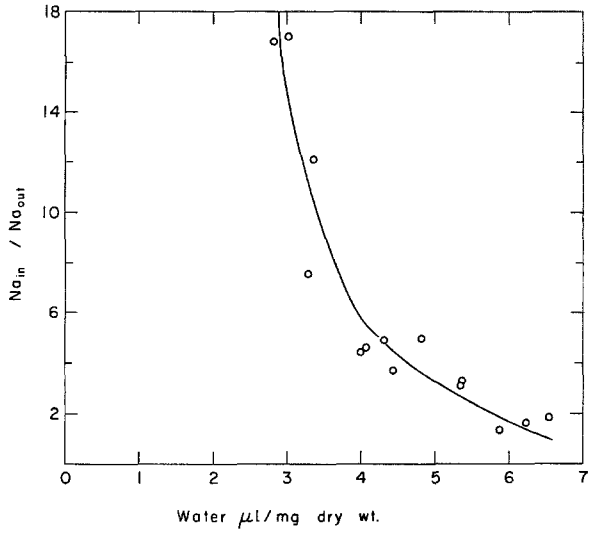

Fig. 8. The correlation between pellet water content and sodium concentration ratio. Conditions are the same as in Table 3 except that more experiments with various external potassium and sodium concentrations are included

possible to use this technique to determine the extent of uptake of various anions. The permeation of various anions was studied previously by passive swelling techniques (Mitchell & Moyle, 1969*b*; Chappell & Haarhoff, 1967). Here we follow the uptake that is induced not by the high concentration of anion but by the active transport process. It is possible to follow this uptake by following the swelling as done previously. However, we find the potassium uptake to be a more sensitive and reproducible index of the transport process.

Table 4 shows that the extent of potassium uptake is determined by the type of anion present in the medium. The anions tested fall into two groups: those that induce potassium uptake and those that do not. (In the absence of any salt a loss of potassium is observed.) The halogens and bicarbonate belong to this latter group. Among the permeant anions, the multivalent acids seem to be more effective than the monovalent. The phosphate effect is exceptional and probably has to do with the "phosphate swelling" induced by high phosphate concentrations (Rottenberg & Solomon, 1969), since at lower phosphate concentrations potassium uptake is observed. On following potassium uptake in the presence of high phosphate with glass electrodes the initial uptake is followed by a loss so that the net change after a 10-min incubation is very small. The effectiveness of the anions is similar in the absence or the presence of valinomycin, except for succinate

Table 4. Effect of various anions on potassium uptake[a]

Anion (10 mM)	ΔK (nmoles/mg dry wt)	
	−Valinomycin	+Valinomycin
Succinate	35	463
Sulfate	190	317
Oxalate	193	225
Citrate	36	160
Fumarate	52	165
Acetate	18	137
Nitrate	20	98
Bicarbonate	−18	−40
Chloride	−30	−27
Bromide	−54	−24
Iodide	−40	−1
Phosphate	−35	1

[a] The incubation medium contained 208 mM sucrose, 2.5 mM sodium ATP, 0.15 μg/mg protein antimycin. Univalent anions were added as potassium salts (10 mM), while divalent anions were added as 5 mM potassium salt and 5 mM sodium salt. pH 6.6, temp = 30 °C. Incubation time, 10 min. Valinomycin, when present, 0.15 μg/ml.

which supports only moderate uptake in the absence of valinomycin, but is the best anion in the presence of valinomycin.

Relation Between ΔpH, Potassium Distribution and Anion Distribution

Table 5 shows the relationship between ΔpH as measured by the ^{14}C-DMO technique, the potassium and water uptake, and the concentration gradient of potassium and ^{14}C-succinate. It is seen that the addition of valinomycin decreases the ΔpH somewhat together with an increase in potassium and water uptake but the potassium gradient is unchanged. The succinate ratio is close to that expected from Model II which predicts that $A^{=}_{in}/A^{=}_{out} = (H^{+}_{out}/H^{+}_{in})^2$. Adding cyanide, reduces both ΔpH and potassium and water uptake, yet the succinate ratio remains consistent with the prediction of Model II. In the presence of oligomycin, potassium uptake is relatively small but ΔpH is unchanged and the succinate ratio is in agreement with the prediction. In the presence of inhibitors of both energy sources (cyanide + oligomycin) there is almost no potassium uptake but ΔpH is still quite high and the succinate ratio again agrees with the prediction. In the presence of 3 mM phosphate, moderate potassium uptake is observed, the potassium gradient is unchanged but there is almost no ΔpH.

Table 5. Relation between ΔpH, potassium and succinate concentration ratios[a]

Medium	ΔpH	Δ Water (µliters/mg dry wt)	ΔK (nmoles/mg dry wt)	K_{in}/K_{out}	Succinate in/out	Expected succinate ratio
−Valinomycin	0.75	0.30	67	11.0	24.0	31.0
+Valinomycin	0.57	2.20	270	11.0	12.0	14.0
+Valinomycin +CN	0.41	0.96	81	12.0	6.4	6.6
+Valinomycin +oligomycin	0.54	0.35	75	10.5	14.0	12.0
+Valinomycin +oligomycin +CN	0.48	0.20	15	11.5	7.0	8.7
+Valinomycin +PO_4 (3 mM)	0.05	3.50	65	10.4	2.0	1.3

[a] Mitochondria were incubated for 20 min in 200 mM sucrose, 10 mM KCl, 5 mM sodium-succinate, 2.5 mM sodium ATP, 1 mM DMO and 0.15 µg/ml valinomycin. Cyanide when added was 1 mM and oligomycin when added was 2 µg/mg protein. Mitochondrial content, 3 mg protein/ml. 95% O_2 − 5% CO_2 was bubbled through the solution. pH 6.5; temp = 30 °C; sucrose space determined by ^{14}C-sucrose distribution, ΔpH from ^{14}C-DMO distribution and succinate ratio from ^{14}C-succinate distribution.

Several conclusions can be drawn from Table 5: (*i*) There is no correlation between the extent of potassium uptake and ΔpH or the succinate concentration ratio; there is no correlation between the potassium concentration ratio and the succinate concentration ratio or the ΔpH; (*ii*) There is a very good correlation between the ΔpH and the succinate concentration; (*iii*) There is probably a fairly good correlation between the extent of potassium uptake and succinate uptake. The latter quantity can be estimated from the inner succinate concentration multiplied by the water content; with valinomycin succinate uptake is estimated as 130 nmoles/mg dry wt compared with 270 for potassium, and with the addition of cyanide, succinate uptake is 32 nmoles/mg dry wt compared with 81 for potassium. (It is expected that two ions of potassium are accumulated with each succinate.)

In Table 6 various other anion distributions are compared with ΔpH and potassium uptake; in this study all systems include valinomycin and also antimycin to inhibit respiration. It is seen that succinic, acetic and oxalic acid all give ratios that are close to those calculated from our model on the basis of the observed ΔpH. In part B of Table 6 an experiment is presented in which DMO and acetic, succinic and oxalic acids are all present in the incubation medium where the labeled compounds ^{14}C-sucrose, ^{14}C-DMO, ^{14}C-acetic acid and ^{14}C-oxalic acid are added to separate samples

Table 6. Relation between ΔpH and the concentration ratios of acetate, succinate and oxalate [a]

Medium	ΔpH	ΔK (nmoles/mg dry wt)	Δ Water (μliters/mg dry wt)	Anion ratio	
				Found	Expected
A. +Succinate	0.63	225	2.10	16.0	18.0
+Acetate	0.77	107	0.70	5.3	5.9
+Oxalate	0.69	118	0.83	30.0	24.0
B. Complete	0.58	203	1.65		
Acetate	—	—	—	3.6	3.8
Succinate	—	—	—	15.0	14.0
Oxalate	—	—	—	17.0	14.0

[a] The medium contained 10 mM KCl, 5 mM sodium ATP, 200 mM sucrose, 0.15 μg/ml valinomycin, 1 mM DMO and 5 mM of the indicated anion in part A. In part B, 5 mM succinate, 2 mM acetate and 2 mM oxalate were incubated. Incubation time 10 min, temp = 30 °C, pH 6.6. Anion ratios were determined from the distribution of ^{14}C-labeled anions and ^{14}C-sucrose space. ΔpH was determined from ^{14}C-DMO as described in the text. The expected values were calculated from the measured pH assuming $A^{-}_{in}/A^{-}_{out} = H^{+}_{out}/H^{+}_{in}$ and $A^{=}_{in}/A^{=}_{out} = (H^{+}_{out}/H^{+}_{in})^2$.

and the experiments are run in parallel under identical conditions. In this experiment as well very good agreement exists between the ion distribution and the value predicted from the measured ΔpH. In addition, it is observed that $A^{=}_{in}/A^{=}_{out} \simeq (A^{-}_{in}/A^{-}_{out})^2$ as predicted by both Models I and II.

Discussion

The main purpose of this work was to test the hypothesis that there is in the mitochondria, an inward-directed cationic pump which is driven by metabolic energy. We have compared the uptake of potassium in the presence and absence of valinomycin, assuming that such a pump must be stimulated by the antibiotic. We also compared the transport of potassium and sodium in the presence of gramicidin assuming that a cationic pump should cause, under certain conditions, competition between the two cations which would result in different "static head".

The observations that the potassium concentration ratio is the same in the presence and absence of valinomycin, and that potassium and sodium concentration ratios are identical in the presence of gramicidin regardless of the external cation concentrations indicates that these antibiotics only accelerate equilibration but do not stimulate pumping against an electrochemical gradient. Likewise, the observation that valinomycin reduces the

potassium flux ratio to a value which is very close to 1 points to the same conclusion. (This latter observation does not agree with Harris, Catlin & Pressman's (1967) interpretation of their experiments on ^{42}K uptake.)

The results are compatible with Model II in which the cation transport is caused by the formation of membrane potential by the electrogenic proton pump. This conclusion derives strong support from the studies of Skulachev and associates on the distribution of various truly permeable anions and cations in mitochondria. (For review *see* Skulachev, 1971.)

The study of the relation between ΔpH, anion distribution and potassium transport give further support to this conclusion. It was already demonstrated that metabolic anion distribution is governed by ΔpH (Quagliariello & Palmieri, 1970) even though the actual transport process of most of these anions is mediated by exchange carriers (Klingenberg, 1970). Here it is demonstrated that acetate and oxalate, which are not transported by these carriers and succinate are all driven by ΔpH, and although they are co-transported with potassium they are not directly coupled to the potassium transport and their concentration gradient is not related to the potassium gradients.

In conclusion, it seems, that the only ion that is transported and forms energy-dependent electrochemical gradients is the proton which indicates the operation of an electrogenic proton pump.

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