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Ion Transport in Liver Mitochondria. V. The Effect of Anions on the Mechanism of Aerobic K^+ Uptake*

C. Rossi, A. Scarpa, and G. F. Azzone

ABSTRACT: The effect of succinate on the stoichiometry of proton translocation and on mitochondrial swelling during K^+ uptake has been studied. The decrease of the H^+:K^+ ratio from 1 to 0.2 is accounted for quantitatively by the intramitochondrial accumulation of succinate.

When the accumulation of succinate is inhibited at alkaline pH the H^+:K^+ ratio increases. When mitochondria oxidize endogenous substrate the decrease of the H^+:K^+ ratio is due to the reuptake of endogenous anions released during storage. Mitochondrial swelling parallels the accumulation of succinate. When K^+ is taken up by the mitochondria in the absence of anions no swelling is observed. The binding of K^+ to the mitochondria in the absence of anions is dependent on metabolism.

It is generally agreed that a rapid and large uptake of univalent cations and release of protons occurs after addition of valinomycin or gramicidin to liver mitochondria incubated in media supplemented with univalent cations under aerobic conditions. The uptake of cations is accompanied by a reversible mitochondrial swelling. However several problems remain still unsolved, among which are the effect of the anions on the stoichiometry of proton translocation and on the mitochondrial swelling and the identification of the energy-dependent step for cation uptake.

Moore and Pressman (1964) and Chappell and Crofts (1965) reported that stimulation of respiration and swelling occurred only in the presence of anions such as phosphate and arsenate. Azzi and Azzone (1965, 1966), Azzone and Azzi (1966), and Ogata and Rasmussen (1966), on the other hand, observed stimulation of respiration and swelling also in the absence of phosphate when succinate or acetate were present. According to Pressman (1965) the uptake of cations did not account for the osmotic movement of water, whereas a correlation between ion and water movements was observed in other laboratories (Chappell and Crofts, 1966; Azzi and Azzone, 1966; Azzone and Azzi, 1966; Ogata and Rasmussen, 1966).

Moore and Pressman (1964) reported a H^+:K^+ ratio of 0.7–0.9, whereas Azzi and Azzone (1966) reported a H^+:K^+ ratio of 0.25. Both results were obtained in media devoid of anions (added) such as phosphate or acetate. Lynn and Brown (1966) and Ogata and Rasmussen (1966) have reported accumulation of anions during K^+ uptake. Harris *et al.* (1966) also observed a H^+:K^+ ratio as low as 0.2 at pH below 7, whereas the ratio was higher at alkaline pH. Chance and Mela (1966a,b) have proposed that the Ca^{2+} translocation across the membrane is dependent on energy supply and not the binding of Ca^{2+} to the mitochondrial membrane.

In the present report it will be shown that the H^+:K^+ ratio decreases from 1 to 0.1 owing to the influx of anions. The anions take up are either those added to the medium, such as succinate or acetate, or those released from the mitochondria during storage. When K^+ is taken up by the mitochondria together with anions an osmotic uptake of water is observed. The binding of K^+ to the mitochondrial membrane, under conditions where the K^+ is not osmotically active, is dependent on energy supply.

Experimental Section

Rat liver mitochondria prepared in 0.25 M sucrose–0.5 mM EGTA[1] were used throughout and all the experiments were conducted at 20–22°. K^+ accumulation and pH were monitored continuously by means of Beckman 39047 and 39030 combination electrodes, respectively (Azzi and Azzone, 1966; Rossi *et al.*,

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[1] Abbreviations used: EGTA, ethylene glycolbis(β-aminoethyl ether)-*N,N*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

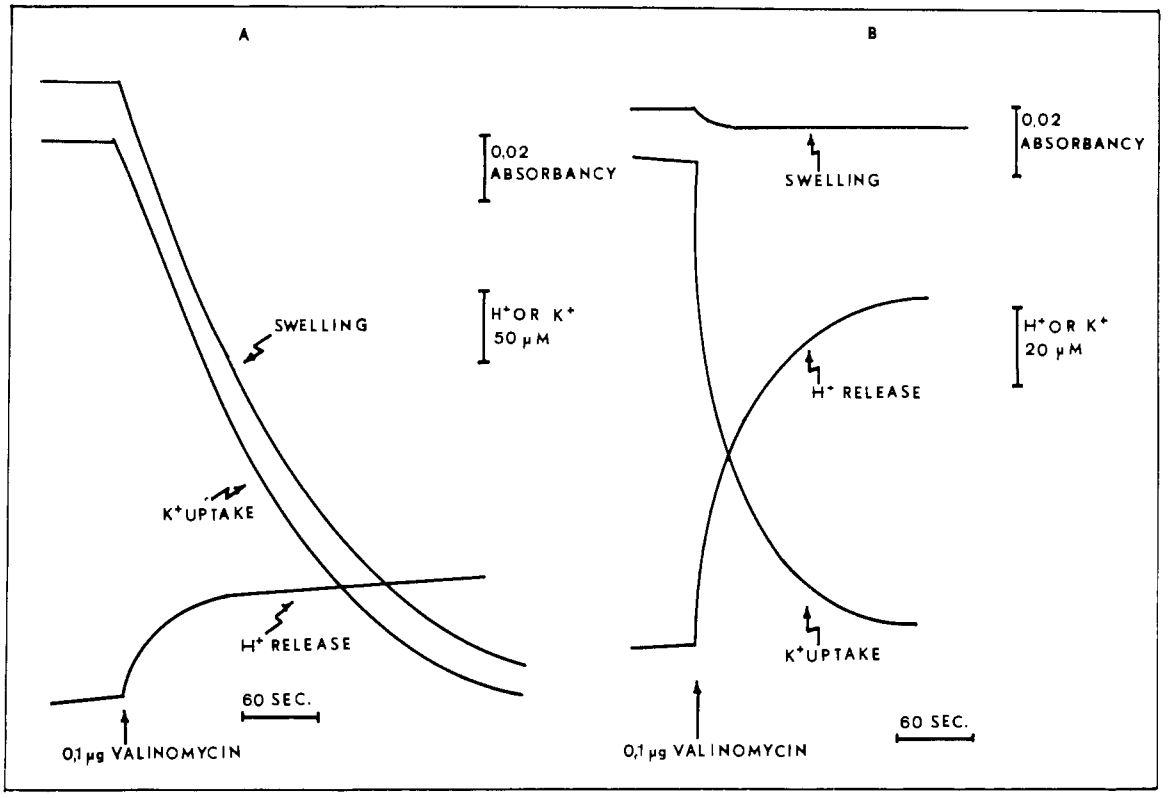

FIGURE 1: H^+ release, K^+ uptake, and swelling in presence and absence of succinate. Experimental conditions were as follows: 250 mM sucrose, 1.3 mM KCl, 3.3 mM Tris-HCl (pH 7.5), and liver mitochondria corresponding to 17.2 mg of protein. In part A 3.3 mM sodium succinate and 2 μM rotenone were also present; final volume, 3 ml; temperature, 22°. The reaction was started by the addition of 0.1 μg of valinomycin.

1967a). Calibration of the K^+ electrode was made in each experiment by adding an amount of KCl solution exactly corresponding to the K^+ taken up by the mitochondria. In many experiments the uptake of cations was measured in parallel samples with the cationic electrode and ^{86}Rb as a tracer. Radioactivity measurements were carried out on both the supernatant and the mitochondrial pellet dissolved in 1 N formic acid. Uptake of succinate was measured in parallel samples with [1-4-^{14}C]succinate (Rossi *et al.*, 1967b). The movement of chloride was measured with $^{36}Cl^-$. In all experiments uptake of cations and release of protons were measured simultaneously. When necessary, optical density measurements were also carried out simultaneously with those of protons and of cations. Inorganic phosphate was measured according to the method of Lindberg and Ernster (1956). Oxygen uptake was measured polarographically with a Clark electrode.

Results

It is seen in Figure 1A that addition of valinomycin to mitochondria incubated in 0.25 M sucrose, 1.6 mM KCl, and 3.3 mM succinate, resulted in an abrupt stimulation of proton release which rapidly declined whereas the K^+ uptake continued for about 4 min. H^+ release was 19.2 μmoles/g of protein and K^+ uptake 138 μmoles/g of protein. Parallel to the uptake of K^+ there was a large mitochondrial swelling.

As shown in Figure 1B addition of valinomycin to mitochondria, incubated in the same medium but oxidizing only endogenous substrates, resulted also in a rapid stimulation of H^+ release and of K^+ uptake. However a close parallelism was observed between H^+ release and K^+ uptake, the two processes levelling off almost simultaneously. H^+ release was 16.3 μmoles/g of protein and K^+ uptake 23.4 μmoles/g of protein. No mitochondrial swelling was observed. Addition of respiratory chain inhibitors abolished both K^+ uptake and H^+ release. It is seen in Figure 2 that addition of valinomycin to mitochondria oxidizing endogenous substrates caused a stimulation of the respiration parallel to the stimulation of K^+ uptake. Rotenone abolished the stimulation of respiration and caused a release of K^+. Respiration and K^+ uptake were again activated by succinate. FCCP caused further stimulation of respiration and K^+ release until anaerobiosis was reached.

Stoichiometry of Proton Release and K^+ Uptake in the

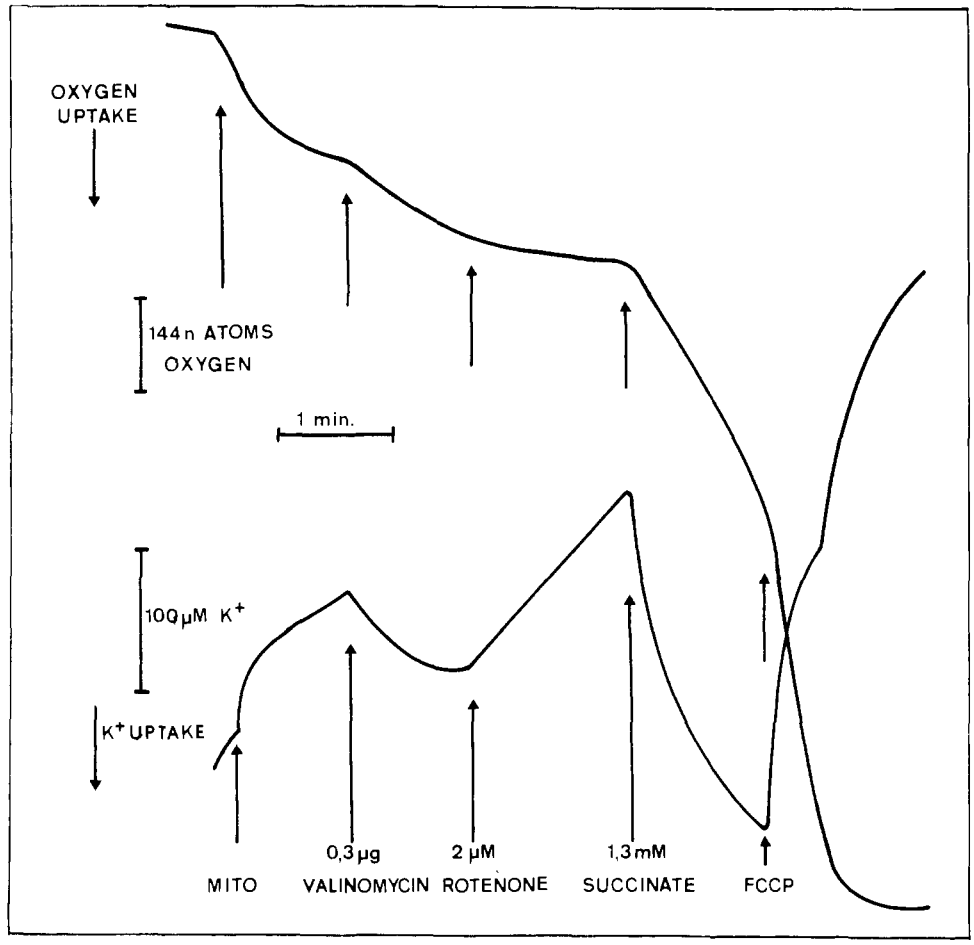

FIGURE 2: Effect of valinomycin, rotenone, succinate, and uncouplers on K^+ uptake and respiration. The medium contained in 3 ml: 0.25 M sucrose, 1.6 mM KCl, 8.8 mM Tris-HCl (pH 7.4), and liver mitochondria corresponding to 9.9 mg of protein.

Presence of Succinate or Acetate. As shown in Figure 3 the extent of K^+ uptake was already maximal at 2 mM KCl, whereas the proton release increased parallel to the increase of the KCl concentration. Above 2 mM KCl, the K^+ uptake was independent of the KCl concentration, and several times higher than the proton release.

The kinetics of K^+ uptake and of H^+ release were not related and the H^+:K^+ ratio tended to decrease while the K^+ uptake was proceeding. As seen in Figure 4 the H^+:K^+ ratio was about 1 after 15 sec and dropped to 0.2 after 2 min.

In Figure 5A it is seen that the extent of proton release was constant at the various succinate concentrations, whereas the amount of K^+ uptake increased from about 60 μmoles/g of protein at 250 μM succinate to 170 μmoles/g of protein at 2.7 mM succinate. In this experiment about 170 μmoles/g of protein was taken up at 1.3 and 2.6 mM succinate in respect to 180 μmoles of K^+ under identical conditions in the experiment of Figure 3. In order to decide whether succinate stimulated the uptake of K^+ because it supplied energy or neutralizing charges or both, succinate was replaced with acetate. As shown in Figure 5B addition of acetate to liver mitochondria oxidizing endogenous substrates, resulted in an increase of K^+ uptake from 40 μmoles/g of protein in the absence of acetate to 260 μmoles/g of protein in the presence of 40 mM acetate. The release of protons was diminished at increasing concentrations of acetate (Rasmussen *et al.*, 1965). In Figure 6 it is seen that addition of acetate caused both an increase of the rate of K^+ uptake and of the respiration. The ratio K^+:$\sim$ remained constant in the range of 3 at the various acetate concentrations.

In the experiment reported in Figure 7 the uptake of $^{86}Rb^+$ was measured simultaneously to the uptake of succinate. The release of protons was measured in a parallel sample. The increase of pH from 6.5 to 8.5 resulted in a large decrease of the amount of succinate and of $^{86}Rb^+$ taken up by the mitochondria. The release of protons, on the other hand, was almost double at pH 8.5 in respect to pH 6.5. Thus the H^+:Rb^+ ratio was influenced by the amount of succinate

TABLE I: Effect of pH on the Release of H^+ and on the Uptake of Cations and Succinate.[a]

pH	H^+ Release	K^+ Uptake	^{86}Rb Uptake	[^{14}C]Succinate Uptake	^{36}Cl Uptake	H^+:K^+
6.5	16.2		128	43.4	2	
6.62	18.3	141	135	47.7	2	0.13
6.99	19.5	147		53.5	2	0.13
7.5	26.0	81.3	83.7	27.5	2	0.32
7.67	28.0	83.0	73.2	24.2	2	0.34
8.5	35.2	39.7	42	11.3	2	0.88
8.8	33.7	32.9				1.05

[a] Experimental conditions as in Figure 1A. H^+ release and K^+ uptake were measured in one sample and the uptake of $^{86}Rb^+$, of [1–4-^{14}C]succinate, and of $^{36}Cl^-$ in another. Values reported were obtained after 4-min incubation. Mitochondrial protein was 16.5 mg. Radioisotope measurements were carried out both on the supernatants and the pellets. The pellet values were slightly lower than the supernatant values and only the latter values are reported in the table. Data are given in micromoles per gram of protein.

taken up, being high without, and low with, succinate accumulation.

The effect of pH on the stoichiometry of K^+ uptake in the presence of succinate is analyzed in Table I. H^+ release and K^+ uptake were measured in one sample with the electrodes, whereas Rb^+, succinate, and Cl^- uptakes were measured in a parallel sample with radioisotopes. The data of K^+ uptake, as measured electrochemically, were very close to the radioisotopic measurements of $^{86}Rb^+$ uptake. The increase of pH had opposite effects on the H^+ release and K^+ uptake, being the former increased and the latter decreased at the higher pH. The decrease of K^+ and Rb^+ uptake was correlated with a large decrease of succinate uptake. No uptake of $^{36}Cl^-$ was observed during succinate uptake. The H^+:K^+ ratio increased from 0.13 at pH 6.5 to 0.8 at pH 9.

The increase of the H^+:K^+ ratio occurring at the alkaline pH was paralleled by a decrease of the extent of mitochondrial swelling (Figure 8). Thus a large swelling was observed when K^+ was taken up together with succinate and with a low H^+:K^+ ratio and a slight or no swelling was observed when K^+ was taken up without succinate with a H^+:K^+ ratio of 1.

A large number of experiments were carried out where the stoichiometry H^+:K^+ or H^+:Rb^+ was measured parallely to the uptake of succinate. In most cases the amount of succinate (or other anions) taken up was able to account for the difference between H^+ ejected and cation taken up. The H^+:cation ratio corrected

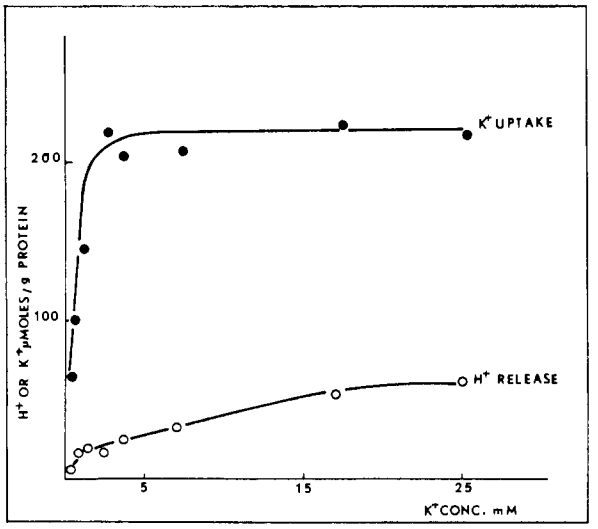

FIGURE 3: H^+ release and K^+ uptake at various K^+ concentrations. Experimental conditions were as in Figure 1A except that succinate was 2.6 mM and mitochondrial protein was 12 mg. Values for H^+ release were calculated on the maximal extent of release after addition of valinomycin.

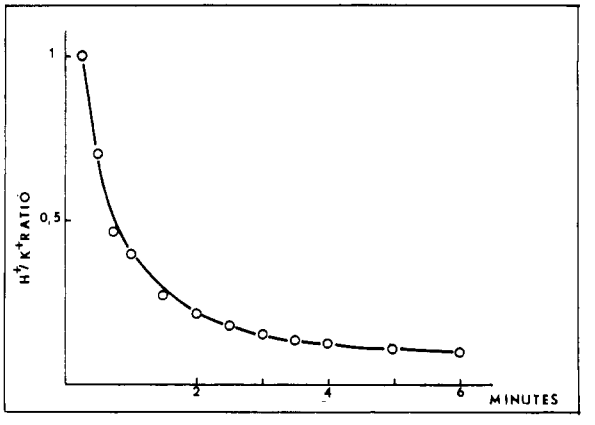

FIGURE 4: H^+:K^+ ratio after various times of incubation. Experimental conditions were as in Figure 1A.

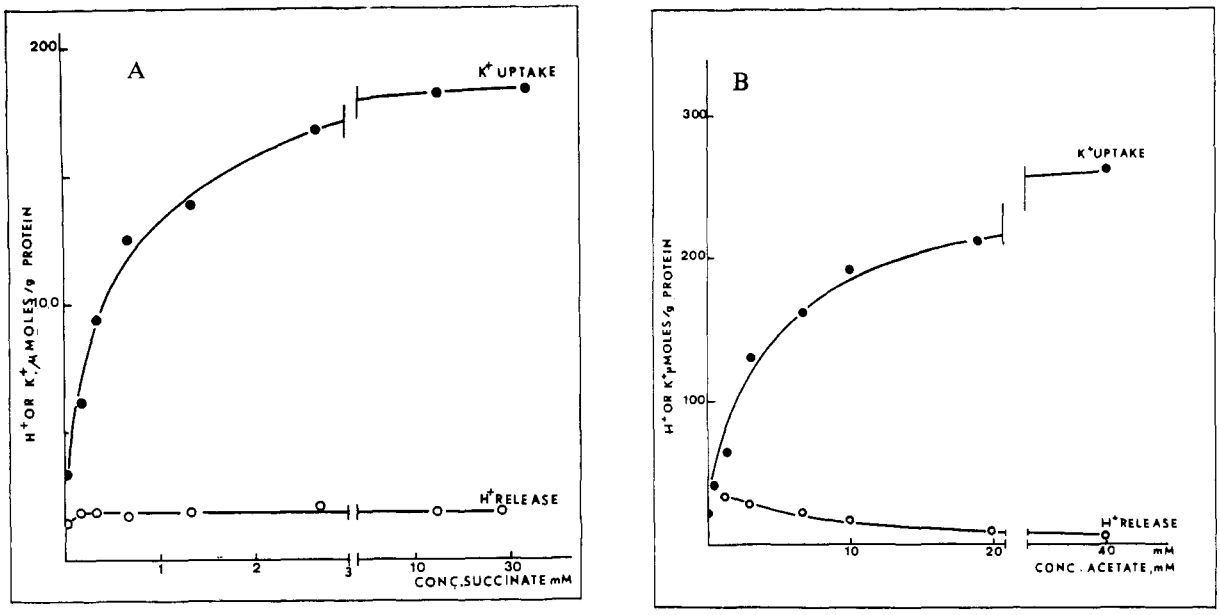

FIGURE 5: H^+ release and K^+ uptake at various succinate and acetate concentrations. Experimental conditions were as in Figure 1A except that mitochondrial protein was 12 mg. The values for K^+ uptake were obtained 5 min after the addition of valinomycin. In Figure 5B KCl was 1.6 mM, and succinate and rotenone were omitted. The sodium salts of succinate and acetate were used.

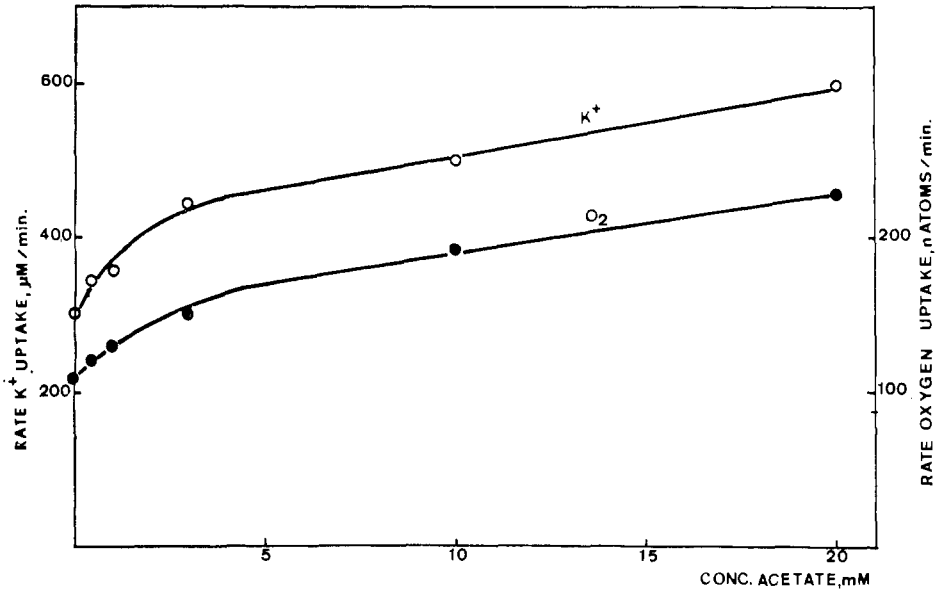

FIGURE 6: Effect of various acetate concentrations on K^+ uptake and respiration. The medium contained in 3 ml: 0.25 M sucrose, 8.8 mM Tris-HCl (pH 7.4), 0.2 μg of valinomycin, 2 mM KCl, and liver mitochondria corresponding to 22 mg of protein. Acetate was used as sodium salt.

for the uptake of succinate was constantly in the vicinity of 1.0.

Stoichiometry of Proton Release and K^+ Uptake in the Absence of Succinate. The H^+:K^+ ratio in mithocondria oxidizing endogenous substrate was found to vary from 0.5 to 1. Among the factors found to affect the H^+:K^+ ratio were the pH of the medium and the storage of the mitochondria. As shown in Figure 9, the H^+:K^+ ratio was lower at pH 7 and tended to increase both at the acidic and the alkaline pH. At the lowest H^+:K^+ ratio a slight swelling became apparent, which however disappeared at pH 8.0

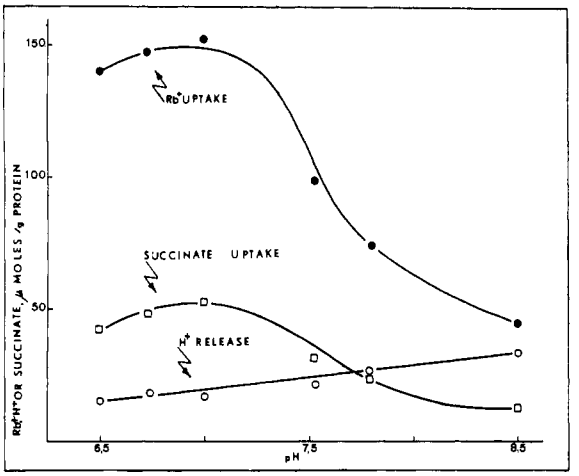

FIGURE 7: H^+ release and Rb^+ and succinate uptake at various pH. Experimental conditions were as in Figure 1A except that concentration of [1-4-^{14}C]succinate was 2.6 mM and mitochondrial protein was 15.7 mg.

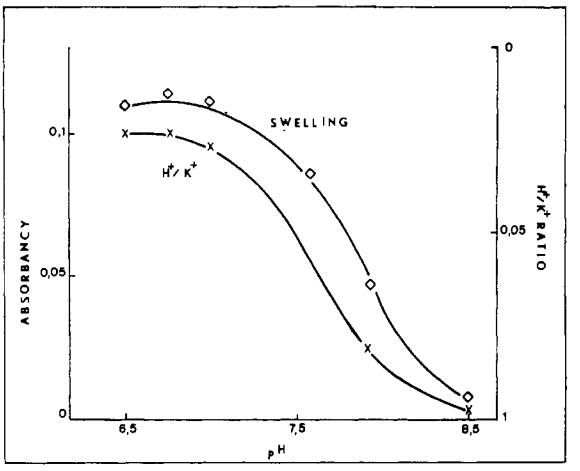

FIGURE 8: Relationship between decrease of H^+:K^+ ratio and increase of mitochondrial swelling at various pH. Experimental conditions were as in Figure 1A except that mitochondrial protein was 18.3 mg for measurements of H^+:K^+ ratio and 6.1 mg for measurements of absorbancy.

and 6.5. It is also seen in Figure 9 that a good parallelism was found between the effect of pH on the H^+:K^+ ratio and on the P_i uptake. The P_i measured was that released from the mitochondria. Apparently the mitochondria release anions during storage and these anions are taken up again together with K^+ thereby lowering the H^+:K^+ ratio from 1 to 0.5. No uptake of P_i was observed at pH 8.5 and the H^+:K^+ ratio was close to 1 (Figure 9). Further support to this suggestion is given by the experiment of Figure 10. The H^+:K^+ ratio dropped during storage from 1 to 0.5 parallel to a release of P_i from the mitochondria. However the H^+:K^+ ratio of washed mitochondria, after various time of storage, was constantly raised again to about 0.9.

H^+ release and K^+ uptake in the absence of succinate were dependent on the concentration of KCl in the medium as shown in Figure 11. Above 5 mM KCl, the amount of K^+ taken up tended to decrease. The rate of K^+ uptake increased from about 50 μmoles of K^+/min per g of protein at 1 mM KCl in the medium, to about 90 μmoles of K^+/min per g of protein at 10 mM KCl. Parallely the rate of respiration increased from 4.8 μatoms/min per g of protein at 1 mM KCl to 8.4 μatoms/min per g of protein at 10 mM KCl. The time curve of H^+ release was biphasic, and after an initial large release a lower steady state was attained. Only the steady-state values are reported in the figure.

In Figure 12 it is seen that the binding of K^+ was accompanied by oxidation of the mitochondrial pyridine nucleotide. The decrease of fluorescence of the pyridine nucleotide was dependent on the concentration of KCl in the medium. In order to exclude that the anions released from the mitochondria could play a role in causing the oxidation of the pyridine nucleotide, the experiment was repeated at pH 8.5 where the H^+:K^+ ratio is 1 and no anion uptake is observed. As seen in Figure 13 a decrease of fluorescence was observed after addition of 1 mM KCl, and not after 1 mM NaCl, to valinomycin-treated mitochondria. Uptake of K^+, release of H^+, and oxidation of pyridine nucleotide, reported in the experiments of Figures 11–13, were abolished by rotenone (*cf.* also Figure 2).

Discussion

Accumulation of succinate within the mitochondria during cation uptake has been observed in several laboratories (Lynn and Brown, 1966; Ogata and Rasmussen, 1966; Rossi *et al.*, 1967b). The effects of the anion accumulation will be discussed below.

The anion influx causes a decrease of the H^+:cation ratio. Rasmussen *et al.* (1965) observed that the H^+:Ca^{2+} ratio was decreased from 1 to 0.2 after addition of 50 mM acetate. Rossi *et al.* (1967b) have reported that accumulation of succinate accounts to a large extent for the decrease of the H^+:Ca^{2+} ratio from 2 to 1. From the results presented here it appears that H^+:K^+ ratio is decreased owing to the parallel influx of succinate and that the H^+:K^+ ratio lies in the vicinity of 1 only when part of the K^+ taken up is accounted for by the uptake of succinate. The present results are therefore at variance from those of Moore and Pressman (1964) who found a H^+:K^+ ratio close to 1 with mitochondria oxidizing succinate. Presumably the high ratio found by Moore and Pressman (1964) was due to a lack of accumulation of succinate under their experimental conditions. In the absence of succinate the H^+:K^+ ratio is decreased because of reuptake of endogenous anions released from the

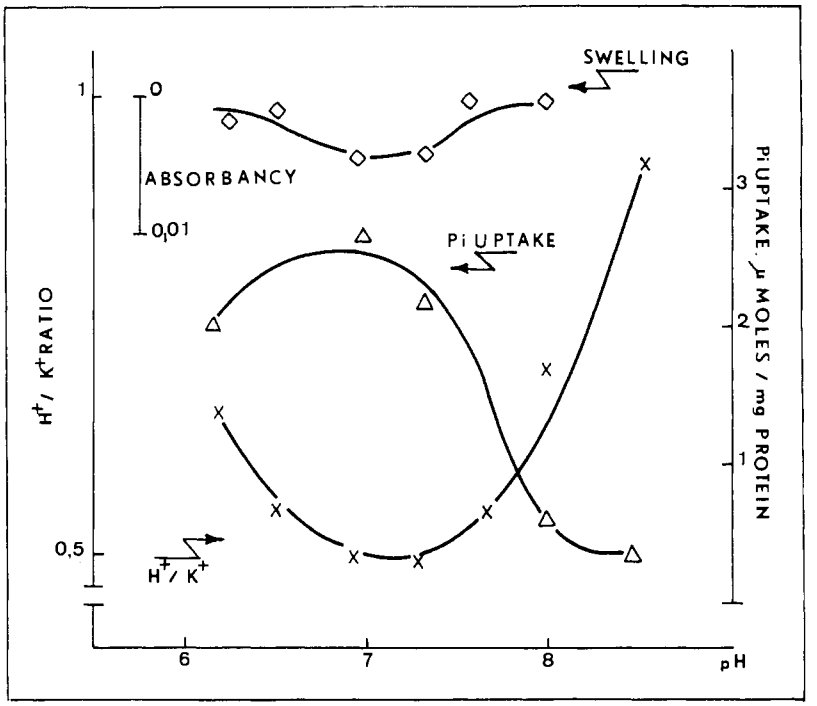

FIGURE 9: Relationship among $H^+:K^+$ ratio, swelling, and uptake of P_i at various pH in the absence of succinate. Experimental conditions were as follows: 250 mM sucrose, 1.3 mM KCl, 3.3 mM Tris-HCl (pH 7.5); mitochondrial protein, 14.3 mg; temperature, 22°; final volume, 3 ml. The reaction was started by the addition of 0.1 μg of valinomycin. Uptake of inorganic phosphate was measured in parallel samples.

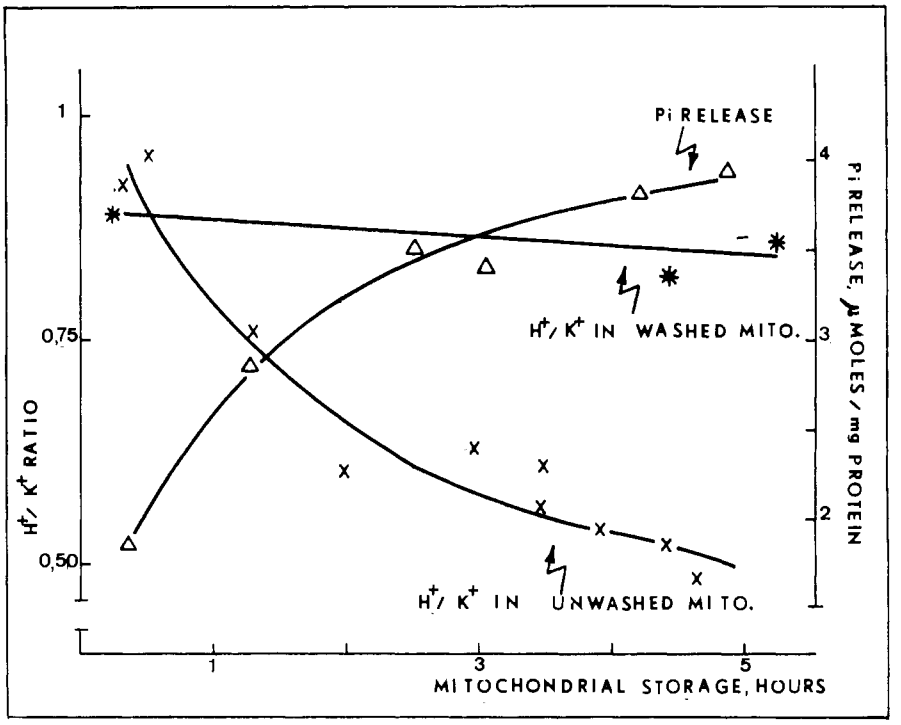

FIGURE 10: Relationship between $H^+:K^+$ ratio and P_i release from the mitochondria during storage. Experimental conditions were as in Figure 8 except that mitochondrial protein was 16 mg. The $H^+:K^+$ ratio was measured either directly with mitochondria stored for variable periods of time at 4° or with mitochondria which had been diluted, centrifuged, and resuspended.

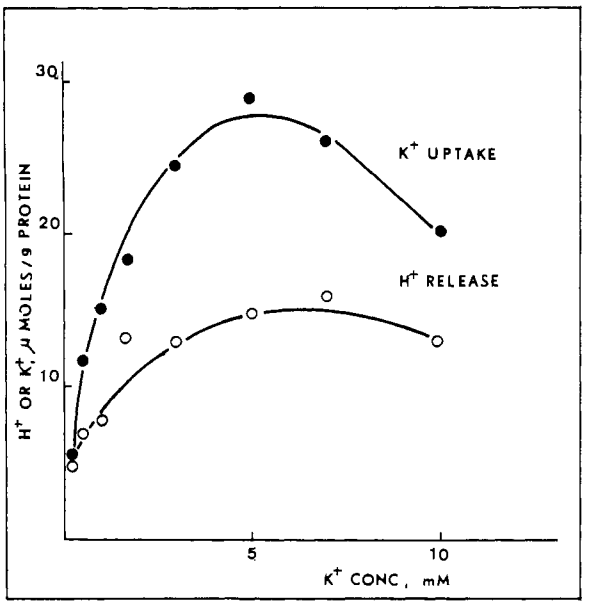

FIGURE 11: H^+ release and K^+ uptake at various KCl concentrations in the absence of succinate. Experimental conditions were as in Figure 8 except that mitochondrial protein was 15 mg.

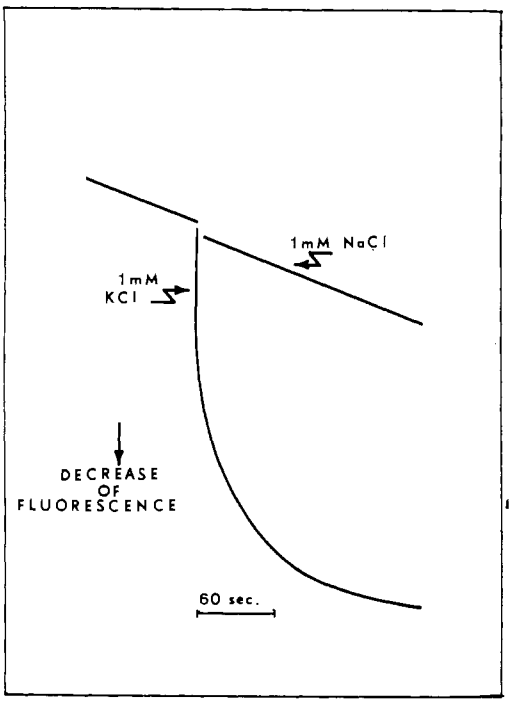

FIGURE 13: Oxidation of mitochondrial piridin nucleotide induced by valinomycin at pH 8.6. Experimental conditions were as in Figure 11 except that the pH was 8.6. Mitochondria were pretreated by 0.05 μg of valinomycin and the reaction was started by the addition of 1 mM KCl as 1 mM NaCl.

mitochondria during storage. Decrease of the H^+:Ca^{2+} ratio during aging has been reported by Rossi *et al.* (1967b). Previous stoichiometries of proton translocation during the uptake of K^+ or Ca^{2+} reported from various laboratories have usually been obtained with mitochondria oxidizing exogenous substrates. The uptake of these substrates, together with the endogenous anions, presumably accounts for the low H^+:cation ratios observed. The present findings thus indicate that electroneutrality is maintained in mitochondria during cation uptake.

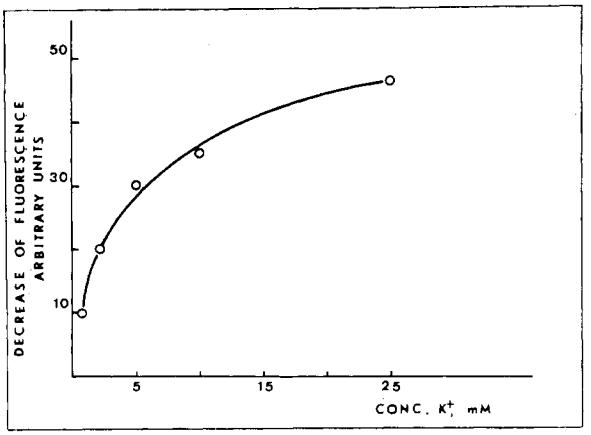

FIGURE 12: Oxidation of mitochondrial piridin nucleotide induced by valinomycin in absence of permeant anions. Experimental conditions were as follows: 250 mM sucrose, 5 mM Tris-HCl at pH 7.5, mitochondrial protein, 5.5 mg; final volume, 2 ml; temperature, 22°. The reaction was started by the addition of 0.05 μg of valinomycin.

The accumulation of the anions was inhibited at alkaline pH. The inhibition concerned various types of anions, namely succinate, phosphate, and acetate. As reported previously the inhibition was observed also during the uptake of Ca^{2+} (Rossi *et al.*, 1967b). When the accumulation of the anions was inhibited there was no decrease of the H^+:K^+ ratio and swelling.

Mitochondrial swelling was observed only when anions entered the mitochondria together with K^+. Ogata and Rasmussen (1966) suggested that the swelling observed in chloride medium could be due to the accumulation of substrate plus the greater dissociation of AK as compared to AH^-, where A^- represents unknown anionic sites within the mitochondria. Our finding are in substantial agreement with those of Ogata and Rasmussen (1966) in that mitochondrial swelling was observed during K^+ uptake parallel to the accumulation of the anion. However since the experiments of Ogata and Rasmussen (1966) were always carried out in the presence of substrate anions, the dependence of mitochondrial swelling on the uptake of anions could not be assessed. Graven *et al.* (1967) have recently shown that under several conditions

uptake and release of K^+ were not followed by mitochondrial swelling and shrinkage and considered the swelling to be more related to the turnover than to the net accumulation of K^+. The experiments reported in the present study where the uptake of K^+ was measured with mitochondria oxidizing only endogenous substrates, suggest that the osmotic activity of the K^+ taken up by the mitochondria is dependent on the influx of permeant anions and further support the osmotic nature of the water movement (Chappell and Crofts, 1965; Rasmussen *et al.*, 1965; Azzone and Azzi, 1966; Ogata and Rasmussen, 1966; Azzi and Azzone, 1966).

A parallelism was always found between H^+:K^+ ratio and mitochondrial swelling, and under conditions where all the K^+ taken up exchanged with H^+, no movement of water was observed. We suggest that the K^+ taken up in exchange with H^+ becomes bound to the anions of the inner mitochondrial membrane and therefore that the H^+ released from the mitochondria during cation uptake is originated to a large extent from the anions of the mitochondrial membrane. This suggestion is also in agreement with the conclusion that the pH-dependent anaerobic binding of Rb^+ also involves a binding of osmotically inactive Rb^+ to the anions of the mitochondrial membrane (Azzone *et al.*, 1967).

Chance and Mela (1966a,b) have proposed a distinction between binding of cations to the membrane and translocation of the cations through the membrane and suggested that only the latter be linked to metabolism. This proposal is not supported by the present data where the binding of osmotically inactive K^+ to the membrane anions was dependent on energy supply as indicated by the shift of the steady state of the pyridine nucleotide toward a higher oxidation (Figures 12 and 13). Presumably the binding of cations to the membrane is an obligatory step in the over-all process of cation accumulation in the mitochondria, although the ionization of the membrane constituents may or may not be dependent on metabolism.

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