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ELECTRONEUTRAL H^+-K^+ EXCHANGE IN LIVER MITOCHONDRIA

REGULATION BY MEMBRANE POTENTIAL

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The paper analyzes the factors affecting the H^+-K^+ exchange catalyzed by rat liver mitochondria depleted of endogenous Mg^{2+} by treatment with the ionophore A23187. The exchange has been monitored as the rate of K^+ efflux following addition of A23187 in low-K^+ media. (1) The H^+-K^+ exchange is abolished by uncouplers and respiratory inhibitors. The inhibition is not related to the depression of ΔpH, whereas a dependence is found on the magnitude of the transmembrane electrical potential, $\Delta\psi$. Maximal rate of K^+ efflux is observed at 180–190 mV, whereas K^+ efflux is inhibited below 140–150 mV. (2) Activation of H^+-K^+ exchange leads to depression of ΔpH but not of $\Delta\psi$. Respiration is only slightly stimulated by the onset of H^+-K^+ exchange in the absence of valinomycin. These findings indicate that the exchange is electroneutral, and that the $\Delta\psi$ control presumably involves conformational changes of the carrier. (3) Incubation in hypotonic media at pH 7.4 or in isotonic media at alkaline pH results in a marked activation of the rate of H^+-K^+ exchange, while leaving unaffected the level of Mg^{2+} depletion. This type of activation results in partial 'uncoupling' from the $\Delta\psi$ control, suggesting that membrane stretching and alkaline pH induce conformational changes on the exchange carrier equivalent to those induced by high $\Delta\psi$. (4) The available evidence suggests that the activity of the H^+-K^+ exchanger is modulated by the electrical field across the inner mitochondrial membrane.

Introduction

The view [1] that a large electrical field, $\Delta\psi$, negative inside, exists across the membrane of energy-transducing systems such as mitochondria and bacteria [2,3] has important physiological implications. If inorganic cations, such as K^+ and Ca^{2+}, were distributed at electrochemical equilibrium with a $\Delta\psi$ of 180 mV, given that their cytosolic concentration is presumably about 150 mM and 1 μM, respectively, their matrix concentration would be 150 M and 1 M, respectively [4–7]. This indicates the need for a mechanism maintaining both K^+ and Ca^{2+} displaced from electrochemical equilibrium, i.e., moving K^+ and Ca^{2+} against their electrochemical gradient. The most obvious candidates for such a process are the electroneutral H^+-K^+ and H^+-Ca^{2+} exchanges, which result in a net efflux of these cations. The concept of H^+/K^+ antiporters has indeed been an integral part of the chemiosmotic hypothesis since its first formulation.

Abbreviations: $\Delta\psi$, membrane potential; $\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_K$, H^+ and K^+ electrochemical gradients; $\Delta\mu_H$ and $\Delta\mu_K$, H^+ and K^+ chemical gradients; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; $TPMP^+$, triphenylmethylphosphonium ion; DMO: 5,5-dimethyl-2,4-oxazolidinedione; pK_o, $-\log[K^+]$ outside the mitochondrial compartment; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, 4-morpholinepropanesulfonic acid.

A variety of experimental approaches has been used during the past 20 years to study the problem of the H^+-K^+ exchange reaction. Mitchell and Moyle [8] explained the mitochondrial swelling in K^+ salts of weak acids as due to an H^+-K^+ exchange, whereas Azzi and Azzone [9] provided evidence for this reaction from measurements of active K^+ extrusion from swollen mitochondria. The group of Brierley [10,11] provided evidence for an electroneutral H^+-K^+ exchanger from $^{42}K^+$ flux measurements. In later studies it has been found that the rate of H^+-K^+ exchange is low in native mitochondria but can be considerably enhanced after modifications of the mitochondrial membrane such as Mg^{2+} depletion [6,12–15] and membrane stretching [6,13]. Garlid [7] has recently proposed a crucial role for Mg^{2+} in the 'Mg^{2+} carrier brake' hypothesis, whereby the rate of H^+-K^+ exchange is controlled by the concentration of free matrix Mg^{2+}. Enhancement of the rate of H^+-K^+ exchange follows: (i) addition of A23187 (Mg^{2+} depletion) [14,15]; (ii) swelling in hypotonic [16] or isotonic media [17] (Mg^{2+} dilution); (iii) addition of Mg^{2+}-complexing anions (Mg^{2+} complexation) [14]. In all these cases the release of the carrier brake control mechanism would be due to the lowering of Mg^{2+} matrix activity [7].

A fundamental question is the driving force of the H^+-K^+ exchange. Present views favor an electroneutral exchange, driven by the chemical gradients of H^+ and K^+, $\Delta\mu_H$ and $\Delta\mu_K$ [5–8,12]. However, the process of A23187-induced K^+ efflux catalyzed by bovine heart mitochondria, thought to occur via the H^+-K^+ exchanger, is inhibited by uncouplers and respiratory inhibitors [15]. This finding has been explained by assuming that both respiratory inhibitors and uncouplers act by decreasing the ΔpH. An outwardly directed K^+ flux, sensitive to uncouplers and respiratory inhibitors, was also observed in 1975 by Diwan and Tedeschi [18], and taken as evidence against the view that K^+ distribution can be explained on the basis of a purely electrophoretic transport [19].

The present investigation aims to analyze the regulation of the H^+-K^+ exchange in A23187-treated mitochondria, and the problem of the uncoupler sensitivity. It will be shown that the rate of A23187-induced K^+ efflux in isotonic media at pH 7.4 is closely related to the magnitude of $\Delta\psi$, an almost exponential rise occurring at $\Delta\psi$ values exceeding 150 mV. Hypotonic swelling and alkaline pH cause a dramatic increase of the rate of K^+ efflux, together with a progressive independence of the rate of K^+ efflux from the $\Delta\psi$ value; then, in mitochondria incubated in isotonic media at pH 8.5 or in hypotonic media at pH 7.4, about 40% of the exchange becomes uncoupler insensitive. It is suggested that the effect of uncouplers and respiratory inhibitors, which is apparently not related to the dimension of ΔpH, is due to a $\Delta\psi$ modulation of the H^+-K^+ exchange carrier. A $\Delta\psi$ modulation of Ca^{2+} efflux has recently been observed in liver mitochondria [20].

Materials and Methods

Rat liver mitochondria were prepared in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM EGTA [21]. The last washing was carried out in an EGTA-free medium, containing 0.5 mg/ml of bovine serum albumin. Mitochondrial protein was assayed with the biuret method, using bovine serum albumin as a standard.

K^+ fluxes were monitored with a K^+-selective eletrode (Schott, Mainz) in a water-jacketed, thermostatically controlled vessel equipped with a magnetic stirrer. Calibration of the electrode was performed by multiple additions of known amounts of KCl before each experiment. When plotted against electrode output readings (or pen deflection on a strip chart recorder) the log of external K^+ concentration gave a straight line, with a slight dependence of the slope on the incubation medium composition. Increase in K^+ concentration due to addition of mitochondria, and subsequent rate of K^+ efflux were calculated by extrapolation on the pertinent calibration line.

Mitochondrial Mg^{2+} content was determined by atomic absorption spectroscopy using a Perkin Elmer 305 B atomic absorption spectrophotometer. Mitochondria were incubated under the specified conditions in 1.6-ml capacity polyethylene centrifuge tubes (1.33 mg of mitochondria in a final volume of 1 ml). At the specified times, mitochondria were centrifuged in a Microcent desk centrifuge (Terzano, Milan) operating at 12000 rpm for 5 min. The supernatants were decanted, the tube walls blotted dry, and each pellet dis-

solved by treatment with 1 ml of a 1 mM NaEDTA solution containing 0.1% (w/w) NaCl and 0.9% (w/w) deoxycholate, at room temperature, for 6–12 h. The dissolved pellets were then assayed for Mg^{2+} content. The kinetics of A23187-induced Mg^{2+} depletion were also studied by dual-wavelength spectrophotometry, by monitoring the absorbance changes of the Mg^{2+} indicator, Eriochrome blue [22], with an Aminco DW 2a dual-wavelength spectrophotometer, equipped with magnetic stirring and thermostatic control (wavelength pair 580 minus 560 nm, 50 μM Eriochrome blue. 1.25 mg/ml of mitochondria, final volume 2 ml, 25°C).

H^+ fluxes were measured with the Aminco DW 2a dual-wavelength spectrophotometer by monitoring the absorbance changes of the pH indicator, phenol red (wavelength pair 560 minus 610 nm, 20 μM phenol red, 1.25 mg/ml of mitochondria, final volume 2 ml, 25°C). In these experiments 0.5 mM Tris was used instead of the usual 10 mM Tris-Mops buffer.

Oxygen consumption was measured with a Clark oxygen electrode (Yellow Springs Instruments, OH) in a closed 2-ml chamber, equipped with magnetic stirring and water-jacket thermostatic control.

Membrane potential, $\Delta\psi$, was calculated on [^{14}C]TPMP$^+$ distribution across the inner mitochondrial membrane [23]. Mitochondria were incubated under the specificed conditions with [^{14}C]TPMP$^+$ (0.03 μCi/ml, corresponding to a final concentration of 20 μM TPMP$^+$). After 1 min of incubation the suspensions were centrifuged at 30000 × g in a Sorvall RC5B refrigerated supercentrifuge for 2 min. The clear supernatants were decanted, the tube wall blotted dry, and the pellets dissolved by treatment with 0.5 ml of the same solubilizer used for Mg^{2+} determinations. The dissolved pellets and 0.1-ml aliquots of the supernatants were added to 4 ml of Packard Insta-Gel scintillation fluid. The radioactivity was assayed with a Packard Tri Carb 300 C liquid scintillation spectrometer and the dpm were calculated using an external standard method and calibration curves to correct for quenching [24]. $\Delta\psi$ was calculated on [^{14}C]TPMP$^+$ distribution, according to the Nernst equation. ΔpH was calculated on [^{14}C]DMO distribution across the inner mitochondrial membrane, according to the method of Addanki et al. [25]. Mitochondria were incubated in the presence of 0.07 μCi/ml of [^{14}C]DMO, and treated exactly as described above for the $\Delta\psi$ measurements.

Mitochondrial volumes were calculated on parallel samples containing ^3H_2O (1 μCi/ml) and [^{14}C]sucrose (0.2 μCi/ml), as described by Zoratti et al. [26]. In hypotonic media the inner mitochondrial membrane becomes permeable to sucrose. The mitochondrial volume was, then, calculated on the basis of the matrix volume found in isotonic media, i.e., assuming that a linear relationship exists between mitochondrial volume and osmolarity^{-1}. This procedure is in accord with the ideal osmometric behavior of mitochondria [27–29]. Unless otherwise stated, all isotopic measurements are the average of duplicate experiments. The incubation media are specified in the figure legends. All chemicals were of analytical grade.

Results

It is now well established that low amounts of the divalent cation ionophore A23187, by depleting endogenous Mg^{2+}, activate an endogenous H^+-K^+ exchanger [12–15]. The A23187 activation is therefore a useful tool to investigate the H^+-K^+ exchange under conditions where, due to the low permeability of the inner membrane to K^+, the electrophoretic K^+ influx is negligible [14].

Fig. 1 shows the kinetics of K^+ efflux induced by A23187 as a function of external pH. K^+ efflux did not occur at pH 6.0, whereas it increased exponentially as the pH was raised above 7.0. It must be emphasized that the A23187-induced K^+ efflux, thought to occur on a putative H^+-K^+ exchanger [7,14,15], is not an electrical K^+ flux down $\Delta\tilde{\mu}_K$, since in all cases addition of excess valinomycin is followed by immediate K^+ reuptake, as shown by Garlid [17].

Fig. 2 shows a similar experiment carried out at pH 7.4 in media of different osmolarity. K^+ efflux was inhibited in hypertonic media, and strongly enhanced in media of decreasing osmolarity. Also in this case, addition of valinomycin determined K^+ reuptake, i.e., the mechanism for K^+ efflux occurring at low osmolarity does not involve a

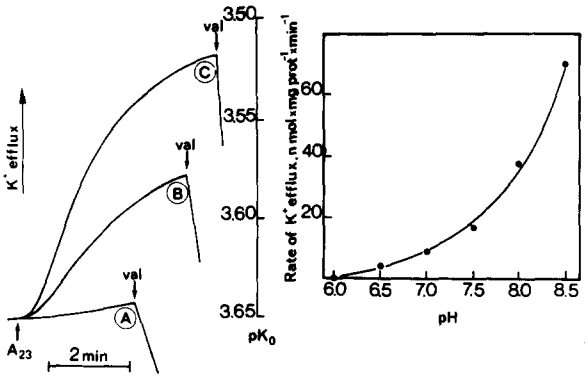

Fig. 1. Effect of pH on rate of K^+ efflux. The incubation medium contained 0.15 M sucrose, 30 mM choline chloride, 30 mM acetate, 2 mM succinate, 0.2 mM EGTA, 2 μM rotenone and 10 mM Tris-Mops adjusted to a final pH of 6.0 (trace A), 7.0 (trace B) or 7.5 (trace C). Total K^+ 0.22 mM, 5.3 mg mitochondria, final volume 4 ml, 25°C. When indicated, 0.72 nmol A23187/mg protein (A_{23}) and 85 pmol valinomycin/mg protein (val) were added. (Right) Quantitative relationship between pH and rate of K^+ efflux following addition of A23187.

pathway driven only by $\Delta\tilde{\mu}_K$. In all cases the A23187-induced K^+ efflux was compensated by H^+ uptake, as judged on the basis of the absorbancy changes of the pH indicator, phenol red (not shown).

It has been shown that the level of intramitochondrial Mg^{2+} is a key factor in the regulation of the activity of H^+-K^+ exchange [7]. It was therefore necessary to ascertain whether the apparent effect of pH and osmolarity on the reaction was due to a different level of Mg^{2+} depletion. Fig. 3 shows that this was not the case, and that within 60–90 s from the addition of A23187 the level of endogenous Mg^{2+} was depressed to almost the same extent under all the conditions tested. The centrifugation method used in these experiments does not allow an accurate kinetic resolution. We therefore followed the kinetics of the A23187-induced Mg^{2+} efflux with the Mg^{2+} indicator, Eriochrome blue [22], with a dual-wavelength spectrophotometer. These experiments indicated that Mg^{2+} efflux is completed within 20–30 s after the addition of A23187 under all the experimental conditions employed in the present study (initial rate of Mg^{2+} efflux was about 80 nmol/mg protein per min, not shown). Thus, beside Mg^{2+} depletion, the H^+-K^+ exchange reaction is independently stimulated by membrane stretching and alkaline pH, as suggested previously [6].

In Fig. 4 the inhibitory effect of Mg^{2+} on K^+ efflux is analyzed. The effects of various Mg^{2+} concentrations were tested at two osmolarities, and the results analyzed according to a Dixon plot; Mg^{2+} behaves as a noncompetitive inhibitor with an apparent K_i of 0.215 mM. Fig. 5 shows the

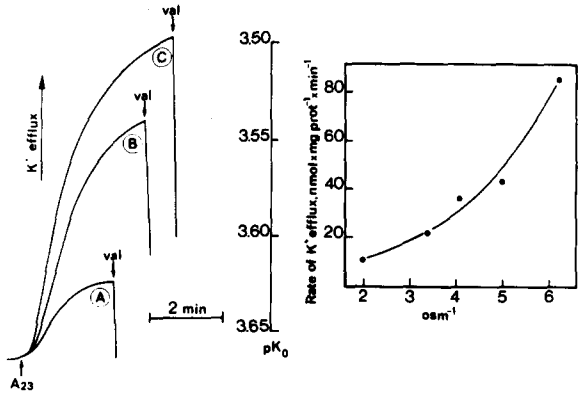

Fig. 2. Effect of osmolarity on rate of K^+ efflux. Experimental conditions as in Fig. 1, except that the final pH was 7.4 and the medium osmolarity was varied by changing the sucrose concentration. Trace A, 0.35 M; trace B, 0.15 M; trace C, 0.1 M. Additions of A23187 (A_{23}) and valinomycin (val) were as in Fig. 1. (Right) Quantitative relationship between medium osmolarity and rate of K^+ efflux following addition of A23187.

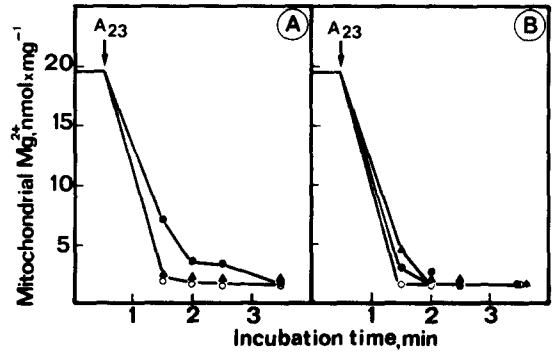

Fig. 3. A23187-induced Mg^{2+} depletion. (A) The incubation medium was the same as in Fig. 1, with a final pH of 6.0 (●——●), 7.0 (○——○), or 8.5 (▲——▲). (B) The incubation medium was the same as in Fig. 2, with sucrose concentrations of 0.35 M (●——●), 0.1 M (○——○) or 0.0165 M (▲——▲). Mitochondria (1.33 mg) were incubated in a final volume of 1 ml, at 25°C. When indicated, 0.72 nmol A23187/mg protein was added, and mitochondrial Mg^{2+} was assayed at various time intervals as described in Materials and Methods.

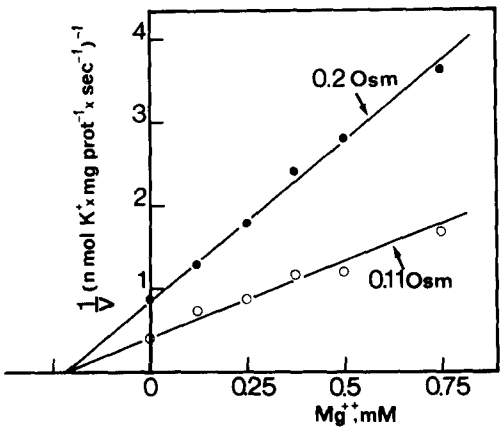

Fig. 4. Kinetics of inhibition of K^+ efflux by Mg^{2+}. The incubation medium contained 30 mM choline chloride, 10 mM Tris-Mops, pH 7.4, 2 mM succinate, 0.2 mM EGTA, 2 mM P_i, 16.5 mM (○——○) or 100 mM (●——●) sucrose, and $MgCl_2$ as indicated. 5 mg mitochondria, final volume 4 ml, 25°C. K^+ efflux was initiated by the addition of 0.76 nmol A23187/mg protein.

effect of weak acids on the rate of K^+ efflux. Increase in the acetate concentration from 0 to 20 mM had a negligible effect. On the other hand, an increase in the P_i concentration from 0 to 1 mM resulted in an almost 3-fold increase, which remained then constant at 2 mM P_i. Since the extent of Mg^{2+} depletion is about equal in the two cases (not shown), it appears that the effect of P_i is presumably at the level of the exchange reaction. However, the possibility of a further depression by P_i of the concentration of the residual free matrix Mg^{2+} cannot be excluded at present. It can be noted that a similar stimulatory effect of P_i has recently been observed for Ca^{2+} efflux [30].

An inhibition of the rate of A23187-induced K^+ efflux by uncouplers and respiratory chain inhibitors has been reported in bovine heart but not in liver mitochondria [15]. Fig. 6 shows that also in rat liver mitochondria both FCCP and malonate had a marked inhibitory effect. Increase in FCCP from 0 to 40 pmol/mg protein resulted in a depression of the rate of K^+ efflux from 180 to about 60 nmol/mg protein per min, and an equivalent inhibition was induced by increase in the malonate concentration from 100 μM to 5 mM (Fig. 6A). These titers of FCCP and malonate are known to cause a significant depression of the proton electrochemical gradient, $\Delta\tilde{\mu}_H$, in State 4.

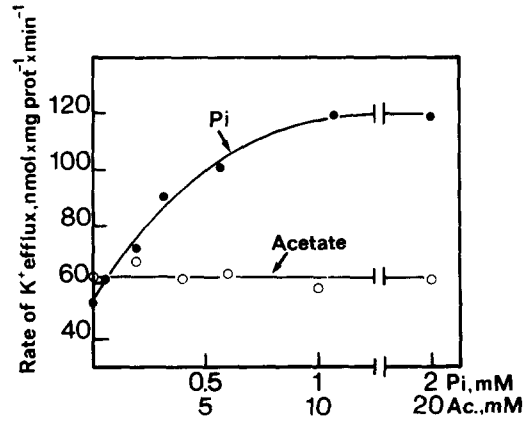

Fig. 5. Effect of phosphate and acetate on rate of K^+ efflux. The incubation medium contained 16.5 mM sucrose, 30 mM choline chloride, 10 mM Tris-Mops, pH 7.4, 0.2 mM EGTA, 0.23 mM total K^+ and P_i (●——●) or acetate (○——○) (Ac.), as indicated. 5 mg mitochondria, final volume 4 ml, 25°C. K^+ efflux was initiated by the addition of 0.76 nmol A23187/mg protein. The 'zero P_i' rate of efflux was determined on mitochondria depleted of endogenous P_i (about 50 nmol/mg protein) by treatment of the mitochondrial stock solution with hexokinase + glucose.

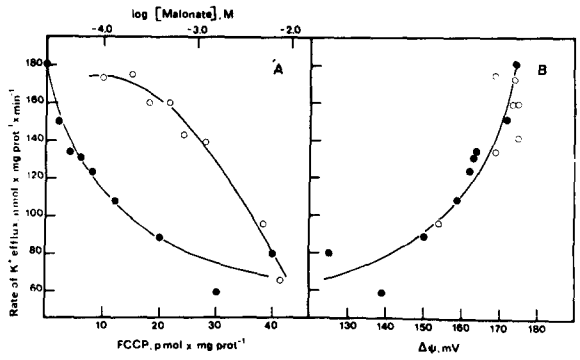

Fig. 6. Effect of uncouplers and respiratory inhibitors on rate of K^+ efflux. Dependence on $\Delta\psi$. (A) The incubation medium was the same as in Fig. 5, with 1 mM P_i and the indicated amounts of FCCP (●——●) or the indicated concentrations of malonate (○——○). 1.25 mg/ml mitochondria, 25°C. Values on the ordinate refer to the rate of K^+ efflux following the addition of 0.76 nmol A23187/mg protein, measured with the K^+-selective electrode (final volume 4 ml). In B the data are replotted as a function of the membrane potential, determined in parallel samples as described in Materials and Methods (final volume 2 ml). (●——●) FCCP, (○——○) malonate.

Since in the presence of P_i the main component of $\Delta\tilde{\mu}_H$ is $\Delta\psi$, the determination of this parameter was also carried out in parallel. Fig. 6B shows that a correlation exists between rate of K^+ efflux and magnitude of $\Delta\psi$. Thus, independently of the nature of the inhibitory effect, the depression of the exchange was a function of the level of $\Delta\psi$ at any given inhibitor concentration.

In Fig. 6 the A23187-induced K^+ efflux occurs at a significant rate even in the presence of uncoupler and malonate, which contrasts with the complete inhibition reported for heart mitochondria [15]. The reason for this discrepancy is analyzed in Figs. 7–9. Fig. 7 shows that the A23187-induced K^+ efflux was completely inhibited by uncoupler in isotonic media at pH 7.0 (Fig. 7A), and that the increase in pH, while enhancing the overall rate of K^+ efflux, caused a decreased sensitivity of the K^+ efflux to uncouplers (Fig. 7B and C). Fig. 8 shows that a similar effect was induced by decreasing osmolarity, indicating that membrane stretching, while increasing the overall rate of exchange, was able to 'uncouple' the reaction from the $\Delta\psi$ control. Similar results were obtained with antimycin A (not shown). Thus, the uncoupler and malonate sensitivity in the experiment of Fig. 6 was only partial because the medium used was hypotonic. This is demonstrated by the experiment shown in Fig. 9, where the rate of K^+ efflux is

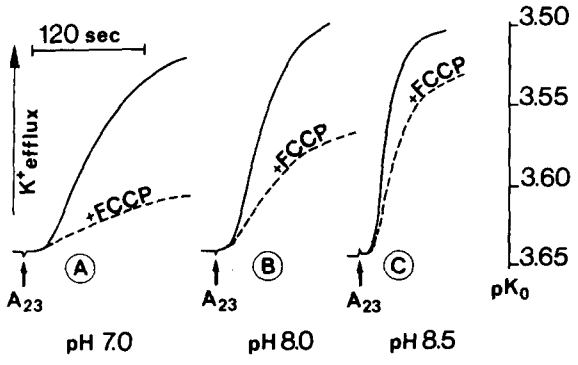

Fig. 7. Dependence of uncoupler sensitivity of rate of K^+ efflux on pH. The incubation medium contained 0.13 M choline chloride, 10 mM Tris-Mops adjusted to pH 7.0 (trace A), 8.0 (trace B) or 8.5 (trace C), 2 mM succinate, 1 mM P_i, 0.2 mM EGTA, 2 μM rotenone. In the dashed traces, 0.2 μM FCCP. 0.23 mM total K^+, 1.25 mg/ml mitochondria, final volume 4 ml, 25°C. When indicated, 0.76 nmol A23187/mg protein was added (A_{23}).

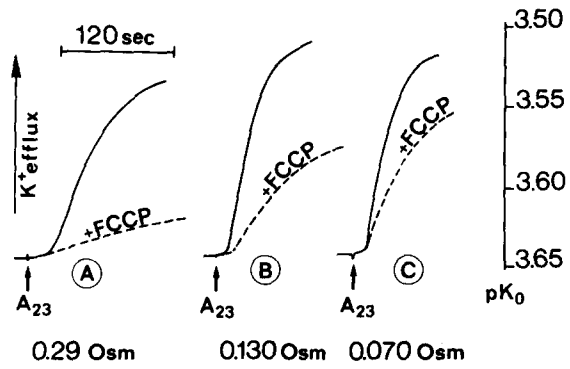

Fig. 8. Dependence of uncoupler sensitivity of rate of K^+ efflux on osmolarity. The incubation medium contained 130 mM (trace A), 50 mM (trace B), or 20 mM (trace C) choline chloride, 10 mM Tris-Mops, pH 7.4, 2 mM succinate, 1 mM P_i, 0.2 mM EGTA, 2 μM rotenone. In the dashed traces, 0.2 μM FCCP. Experimental conditions and addition of A23187 were as in Fig. 7.

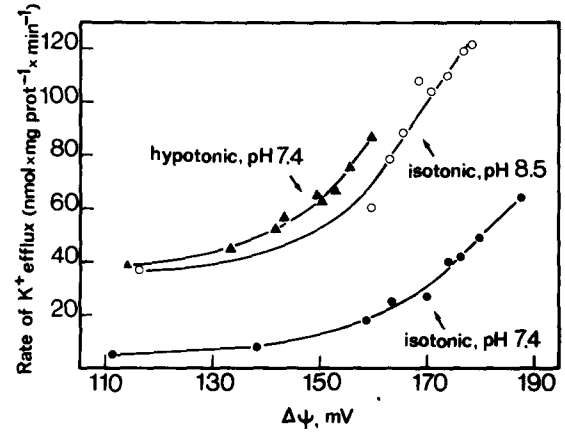

Fig. 9. $\Delta\psi$ dependence of rate of K^+ efflux at various pH and osmolarity. The incubation medium contained 130 mM choline chloride, 10 mM Tris-Mops, 2 mM succinate, 1 mM P_i, 0.2 mM EGTA, 2 μM rotenone, 0.23 mM total K^+, 1.25 mg/ml mitochondria, 25°C. (●——●) pH 7.4, (○——○) pH 8.5, (▲——▲) pH 7.4 and 10 mM instead of 130 mM choline chloride. Membrane potential was varied with amounts of FCCP ranging between 0 and 160 (●——●), 0 and 400 (○——○) or 0 and 80 (▲——▲) pmol/mg protein. Values on the ordinate refer to the rate of K^+ efflux following addition of 0.76 nmol A23187/mg protein, determined with the K^+ electrode (final volume 4 ml). Membrane potential was calculated on parallel samples containing [^{14}C]TPMP$^+$ (final volume 2 ml). For details and further informations see text and Materials and Methods.

correlated with the magnitude of $\Delta\psi$ under different experimental conditions. It can be seen that the overall rate of K^+ efflux was lower, but completely dependent on $\Delta\psi$, in isotonic media at pH 7.4. On the other hand, both alkaline pH and low osmolarity increased the rate of K^+ efflux and, at the same time, were able to uncouple a significant fraction of K^+ efflux from the $\Delta\psi$ control. Titrations of the membrane potential with FCCP revealed that higher amounts of uncoupler were required to obtain the same range of $\Delta\psi$ depression at pH 8.5 with respect to pH 7.4. In contrast, lower amounts of FCCP were necessary in hypotonic as compared to isotonic media (not shown).

In Figs. 6 and 9, the rates of K^+ efflux are measured in A23187-treated mitochondria, while the $\Delta\psi$ assays are carried out in A23187-untreated mitochondria. The advantage of omitting A23187 from the $\Delta\psi$ assays is that it allows the detection of, and the effects of various agents on, the $\Delta\psi$ existing before the alterations due to the H^+-Mg^{2+} or H^+-K^+ exchanges. The possible disadvantage is that the correlation would be misleading if A23187, in addition to induction of H^+-Mg^{2+} and H^+-K^+ exchanges, also affected the $\Delta\psi$. Direct effects of A23187 on the level of $\Delta\psi$ are unlikely in view of the fact that A23187-treated mitochondria, supplemented with ruthenium red or EGTA, maintain respiratory and $\Delta\psi$ levels equal to those occurring in native mitochondria in State 4 [32]. This point is however clarified in the experiment of Table I which shows the values of $\Delta\psi$ and ΔpH before and at different time intervals after the addition of A23187. The values shown in the table are then those of mitochondria in State 4, during A23187-induced K^+ efflux and after termination of K^+ efflux. The level of $\Delta\psi$ was unaffected by initiation and termination of K^+ efflux. On the other hand, the level of ΔpH was depressed considerably during K^+ efflux and restored almost to its initial level after termination of K^+ efflux. The decrease in ΔpH is due to the fact that K^+ efflux occurs in exchange with H^+, resulting in matrix acidification. Thus, during K^+ efflux the ΔpH is very low, and operation of the redox H^+ pumps restores the ΔpH when the exchange has gone to completion. The constancy of $\Delta\psi$ is somewhat surprising, since matrix acidification might be expected to cause a compensatory increase in $\Delta\psi$ similar to that during the H^+-K^+ exchange induced by nigericin. The constancy of $\Delta\psi$ combined with the depression of ΔpH leads to a slight depression of $\Delta\tilde{\mu}_H$, which is accompanied by a slight respiratory stimulation (Fig. 11). This behavior presumably reflects the occurrence of a futile K^+ cycling, limited in rate by the low K^+ permeability. At all events Table I indicates that treatment with A23187 does not cause appreciable variations of $\Delta\psi$ and it is therefore permissible to correlate the rate of K^+ efflux with the level of $\Delta\psi$ in A23187-untreated mitochondria.

TABLE I

CHANGES OF $\Delta\psi$ and ΔpH INDUCED BY A23187

The incubation medium contained 0.13 M choline, 10 mM Tris-Mops, pH 7.4, 2 mM succinate, 1 mM P_i, 0.2 mM EGTA, 2 μM rotenone, 0.2 mM total K^+, [^{14}C]TPMP$^+$ ($\Delta\psi$ measurements) or [^{14}C]DMO (ΔpH measurements). A23187, when present, was 0.76 nmol/mg protein. The experiment was started by the addition of 2.5 mg mitochondria (final volume 2 ml, 25°C). In parentheses is indicated the incubation time after which $\Delta\psi$ and ΔpH were determined. Each value is the average of triplicates ±S.D. For further details see Materials and Methods.

Addition	$\Delta\psi$ (mV)	ΔpH (mV)
None (2 min)	184.2 ± 0.54	34.05 ± 0.43
A23187 (1 min)	184.9 ± 1.13	8.52 ± 0.75
A23187 (6 min)	183.2 ± 1.55	26.7 ± 0.64

Uncouplers are known to decrease both the $\Delta\psi$ and the ΔpH. It is therefore of fundamental importance to assess the role of both components of $\Delta\tilde{\mu}_H$ on the kinetics of H^+-K^+ exchange. Table II shows the effect of low amounts of FCCP and of antimycin A on the rate of K^+ efflux, $\Delta\psi$ and ΔpH. FCCP caused an almost complete inhibition of the rate of K^+ efflux, a 51 mV decrease in $\Delta\psi$, and a 25 mV decrease in ΔpH. Antimycin A shared the effects of FCCP on the rate of K^+ efflux and on the decrease in $\Delta\psi$ (47.5 mV), whereas the effect on ΔpH was significantly lower (10 mV). These data indicate that either the relevant parameter involved in the regulation of the H^+-K^+ exchanger is the $\Delta\psi$ component of $\Delta\tilde{\mu}_H$, or that a 10 mV decrease in ΔpH is sufficient for a 90% inhibition of the exchange. The latter possibility is unlikely for the following reason. Given that the H^+-K^+ exchange is driven by the two H^+ and

TABLE II

COMPARISON BETWEEN EFFECTS OF UNCOUPLER AND ANTIMYCIN A ON RATE OF K^+ EFFLUX, $\Delta\psi$ AND ΔpH

The incubation medium was the same as in Table I. Further additions are listed in the first column (FCCP was 60 pmol/mg protein and antimycin A 0.4 μg/mg protein). 1.25 mg/ml mitochondria, 25°C. The rate of K^+ efflux following addition of A23187 (0.76 nmol/mg protein) was followed with the K^+-selective electrode (final volume 4 ml). The ΔpH and $\Delta\psi$ were determined in parallel samples containing [^{14}C]DMO (ΔpH measurements) or [^{14}C]TPMP$^+$ ($\Delta\psi$ measurements) (final volume 2 ml). Each value is the average of triplicates ± S.D. A23187 was omitted in the $\Delta\psi$ and ΔpH measurements, in order to determine these parameters before the onset of H^+-K^+ exchange. The value of $\Delta\psi$ is, however, unaffected by A23187 (see Table I). For further explanation see text and Materials and Methods.

Addition	Rate of K^+ efflux (nmol/mg protein per min)	$\Delta\psi$ (mV)	ΔpH (mV)
None	47.4 ±0.7	177.53±0.83	30.00±1.58
FCCP	8.16±0.77	126.07±7.2	4.69±1.88
Antimycin A	4.62±0.25	130.6 ±0.68	19.59±0.08

K^+ chemical gradients, $\Delta\mu_H$ and $\Delta\mu_K$, the dimension of the two gradients in a low-K^+ medium is below 20 mV for H^+ (cf. also Table I) and above 180 mV for K^+. Since the major thermodynamic force for the exchange is the K^+ gradient, the control by ΔpH should be kinetic in nature. However, a strict regulatory role of ΔpH is in contrast with the observation that increasing concentrations of P_i, which increases the $\Delta\psi$ component and decreases the ΔpH component of $\Delta\tilde{\mu}_H$ by an extent greater than 10 mV, increase rather than decrease the rate of K^+ efflux via the H^+-K^+ exchanger (Fig. 5).

Fig. 10 further supports the view that the FCCP and antimycin A inhibition of K^+ efflux cannot be explained by their effects on the ΔpH. After addition of FCCP or of antimycin A, A23187 was unable to activate the H^+-K^+ exchanger; yet addition of nigericin was followed by an immediate and extensive K^+ release. Thus, whatever the value of ΔpH in the presence of FCCP plus A23187 or of antimycin A plus A23187, the outwardly directed K^+ chemical gradient can drive K^+ efflux by electroneutral exchange with protons when

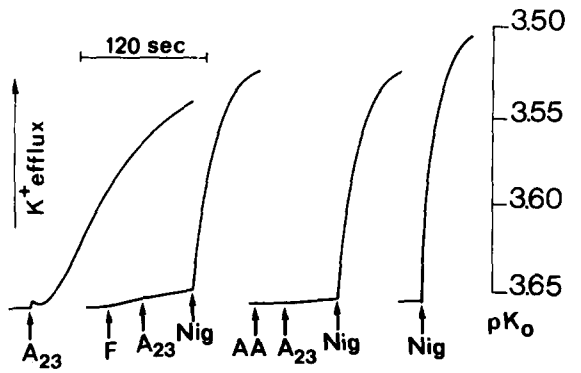

Fig. 10. ΔpH independence of the effect of uncouplers and anitmycin on the H^+-K^+ exchange catalyzed by nigericin and natural carrier. The incubation medium contained 0.13 M choline chloride, 10 mM Tris-Mops, pH 7.4, 2 mM succinate, 1 mM P_i, 0.2 mM EGTA, 2μM rotenone. 5 mg mitochondria, final volume 4 ml, 25°C, 0.22 mM total K^+. When indicated, 0.76 nmol A23187/mg protein (A_{23}), 80 pmol FCCP/mg protein (F), 1 μg antimycin A (AA), 8 pmol nigericin/mg protein (Nig).

catalyzed by nigericin but not when catalyzed by the endogenous exchanger. The simplest explanation of the experiment is that, even in the presence of a large K^+ gradient, the endogenous H^+-K^+ exchanger does not operate at low $\Delta\psi$.

It is well known that the simultaneous presence in mitochondria of two independent transport pathways driven by different thermodynamic forces, as in the case of valinomycin + nigericin for K^+ [31] and of natural carrier + A23187 for Ca^{2+} [32], leads to ion cycling and therefore to energy drain. By analogy, if Mg^{2+} depletion leads to activation of a pathway for K^+ efflux similar in nature to that induced by nigericin, it should be possible to induce a K^+ cycling by increasing the K^+ electrical conductance in A23187-treated mitochondria. The experiment of Fig. 11 indicates that neither A23187 nor valinomycin alone altered significantly the respiration, whereas a steady stimulation was observed when both ionophores were present, independently of the order of addition. Note that when A23187 was added after valinomycin there was a lag phase, reflecting the time necessary to deplete the mitochondria of endogenous Mg^{2+}. The increase in respiration was completely prevented by Mg^{2+}, which inhibits K^+ efflux (Fig. 4).

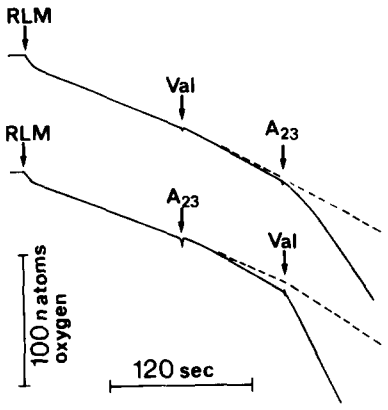

Fig. 11. Stimulation of respiration due to induction of K^+ cycling. The incubation medium contained 0.13 M choline chloride, 10 mM Tris-Mops, pH 7.4, 2 mM succinate, 1 mM P_i, 0.25 mM EGTA, 2 μM rotenone. Total K^+, after addition of mitochondria, was 0.22 mM. Final volume 2 ml, 25°C. When indicated, 2.5 mg mitochondria (RLM), 0.6 μg valinomycin (Val), and 0.76 nmol A23187(A_{23})/mg protein. In the dashed traces, 4 mM $MgCl_2$ was present.

Fig. 12 shows a comparison of the respiratory stimulation induced by increasing amounts of valinomycin in the presence of A23187 (A) or of the electroneutral H^+-K^+ ionophore nigericin (B). In both cases an almost linear relationship was observed, further suggesting that the A23187-induced K^+ efflux occurs by all probability on an

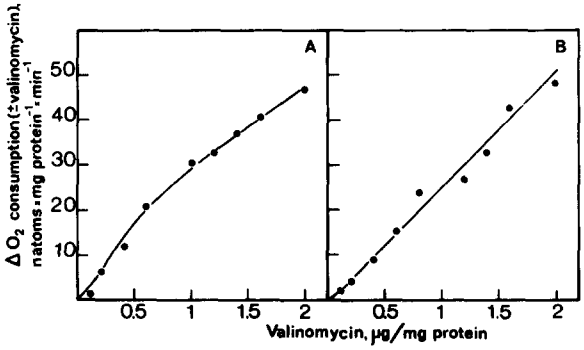

Fig. 12. Stimulation of respiration by K^+ cycling. Comparison between the effects of nigericin and of endogenous H^+-K^+ exchanger. Experimental conditions as in Fig. 11. Mitochondria (2.5 mg) were preincubated for 5 min in the presence of 0.76 nmol A23187/mg protein (A) or of 27 pmol nigericin/mg protein (B). Subsequently, the indicated amounts of valinomycin were added. Values on the ordinate refer to the ΔO_2 uptake, i.e., the difference in rate of oxygen consumption ± valinomycin.

electroneutral H^+-K^+ exchanger, equivalent, with respect to the rate of operation in Mg^{2+}-depleted mitochondria, to approx. 20 pmol nigericin/mg protein.

Discussion

Activation of the exchange

Early experiments on H^+-K^+ exchange were carried out with anaerobic mitochondria in isosmotic media and without Mg^{2+} depletion [8,33]. Subsequent studies, including the present work, have shown that under the above conditions the rate of exchange is negligible, while extensive activation of the exchange may be achieved under several conditions. All the available evidence then indicates that the rate of H^+-K^+ exchange is affected by many different factors ranging from the degree of membrane stretching [6] to the pH (Fig. 1), energy level of mitochondria [15] (Figs. 6 and 9), concentration of free Mg^{2+} [6,7,12,15], presence of a variety of weak acids [14] (Fig. 5) and presumably several others. According to the Mg^{2+} carrier brake hypothesis the H^+/K^+ antiporter is regulated by Mg^{2+}, while other factors would affect the rate of exchange essentially by modifying the concentration of matrix free Mg^{2+} [7].

The present data indicate a more complex regulation, in that the rate of exchange is markedly activated at alkaline pH and at low osmolarity, under conditions of identical Mg^{2+} depletion. A similar effect of alkaline pH [34] on the electrical permeability of the inner membrane for inorganic anions has been previously observed and attributed to alteration of the lipid bilayer structure. The increased exchange activity, however, is presumably not related to this phenomenon, since, even at extremely high pH values addition of valinomycin after A23187-induced K^+ efflux leads to extensive K^+ reuptake, indicating that under these conditions mitochondria retain their functional integrity, i.e., high $\Delta\psi$ (Fig. 1). This holds also for the effect of stretching (Fig. 2), which occurs in a range of osmolarities where there is unfolding of the cristae but no alteration of the membrane ultrastructure [35,36]. Finally, the rate of exchange is depressed by uncouplers or respiratory inhibitors, agents which do not modify directly the concentration of free Mg^{2+}. Conditions

leading to activation of the H^+-K^+ exchange, such as Mg^{2+} depletion or membrane stretching, result also in activation of the H^+-organic cation exchange [37,38]. This observation suggests that the mitochondrial antiporters may be seen as an exchange system interacting with the various ions through forces mainly ionic in nature and unable to distinguish among the various species. A fundamental feature of this exchange system is its high degree of modulation, presumably due to a limited accessibility of the system for the aqueous phases. This property renders the degree of interaction of the antiporter negatively charged sites with the external ion a function of the membrane ionic permeability. Activation of the antiporter by membrane stretching and alkaline pH might reflect the increased ion flow from bulk water to the antiporter-binding sites embedded in the membrane.

Regulation of the exchange

The inhibition of the H^+-K^+ exchange in bovine heart mitochondria by uncouplers and respiratory inhibitors has been interpreted [15] as reflecting the consumption of H^+ during the exchange, and thus the requirement for a continuous regeneration of the ΔpH in order to drive the H^+-K^+ exchange. Several lines of evidence argue against this view:

(1) There is a discrepancy between FCCP and antimycin A with respect to depression of the rate of K^+ efflux on one side and to changes of $\Delta\psi$ and ΔpH on the other. Both FCCP and antimycin A depress to the same extent the rate of K^+ efflux and $\Delta\psi$, whereas ΔpH is depressed by 85% by FCCP and only by 35% by antimycin: yet the rate of K^+ efflux is slightly lower in the latter case (Table II). (2) The rate of $\Delta\mu_H + \Delta\mu_K$-driven H^+-K^+ exchange catalyzed by nigericin is almost unaffected by the same concentrations of FCCP and antimycin A which completely inhibit the exchange activated by A23187 (Fig. 10). (3) The rate of exchange is stimulated by P_i, which is known to depress the ΔpH (Fig. 5). High P_i concentrations which reduce ΔpH to negligible values do not inhibit the exchange. (4) The effect of both uncouplers and respiratory inhibitors is almost immediate [15] (Fig. 10), which is in accord with an effect on a parameter of low capacity, such as the $\Delta\psi$, rather than on one of high capacity, such as the ΔpH. These considerations do not negate a key role of ΔpH as driving force for the H^+-K^+ exchange in vivo, especially in view of the fact that in the living cell the K^+ gradient across the inner mitochondrial membrane is negligible. However, they indicate that the rate of the reaction is not modulated by changes of ΔpH.

The nature of the regulation of H^+-K^+ exchange by $\Delta\psi$ is obscure. On the one hand, the exchange might not be electroneutral: an exchange with a stoichiometry of nH^+/K^+, with $n > 1$, would be strongly affected by the magnitude of the membrane potential. This alternative, however, is in contrast with three observations. First, the level of $\Delta\psi$ is unaffected by the onset of the H^+-K^+ exchange, as well as by its cessation (Table I); second, a charge unbalanced H^+ influx should cause a respiratory increase, proportional to the rate of K^+ efflux and this is not observed upon addition of A23187 (Fig. 11); third, the pattern of the respiratory stimulation following addition of valinomycin is similar when K^+ efflux is catalyzed by nigericin or by the activated native carrier; this indicates that the two K^+-cycling processes are equivalent in nature. The alternative explanation is that the rate of operation of the carrier depends on a particular conformation of the carrier under $\Delta\psi$ control. A regulation of electroneutral exchanges by the membrane energy level seems to be the rule, rather than the exception, in several energy-transducing systems [39–41]. A $\Delta\psi$ dependence of the H^+-Na^+ and H^+-K^+ exchanges has been observed in the case of *Escherichia coli* [42,43]. Furthermore, also the mitochondrial steady-state Ca^{2+} efflux has been proposed to be modulated by the $\Delta\psi$ [20], in analogy with the H^+-Ca^{2+} antiporter of *E. coli* [44].

Our tentative proposal (cf. also Ref. 45) is that the membrane component catalyzing the H^+-K^+ exchange, denoted as C, may exist in an inhibited (C_i) and activated form (C_a), as indicated in the scheme below:

$$C_i Mg^{2+} \underset{\text{pH, stretching}}{\overset{\Delta\psi}{\rightleftharpoons}} C_a Mg^{2+} \overset{Mg^{2+}}{\rightleftharpoons} C_a^* \qquad (1)$$

The transformation of the Mg^{2+}-complexed, inhibited carrier, C_iMg^{2+}, into the Mg^{2+}-complexed, activated carrier, C_aMg^{2+}, can occur as an

effect either of high $\Delta\psi$ or of alkaline pH and stretching. This first step accounts for the fact that the carrier activated either at alkaline pH or after stretching has partially lost its dependence on $\Delta\psi$, i.e., the conformational changes by $\Delta\psi$ are 'equivalent' to those by alkaline pH and stretching. This step also accounts for the fact that the $\Delta\psi$ activation occurs on a carrier where the inhibitory effect of Mg^{2+} has not been removed. The second step, involving the removal of Mg^{2+} and the transformation of the carrier from C_aMg^{2+} into the fully activated form C_a^*, accounts for the fact that whatever the mechanism of carrier activation (high $\Delta\psi$, pH or stretching) the Mg^{2+} depletion always results in a several-fold increase in the exchange.

Physiological role

The present finding that the H^+-K^+ exchange carrier is electroneutral and $\Delta\psi$ modulated may have significant physiological implications. The increased electrical K^+ influx down $\Delta\tilde{\mu}_K$ following the $\Delta\psi$ rise during the State 3–State 4 transition may be exactly balanced by the activation of the carrier catalyzing the H^+-K^+ exchange down $\Delta\mu_H + \Delta\mu_K$. This adjustment would prevent net K^+ accumulation while requiring no change in ΔpH. In turn, the stretching-induced activation may be useful to balance the enhanced K^+ influx due to increased K^+ permeability. It is noteworthy that also Ca^{2+} efflux in energized mitochondria appears to be modulated by $\Delta\psi$ [46]. These data suggest that the $\Delta\psi$ control of electroneutral antiporters could be a general feature of mitochondria and bacteria. Studies on this hypothesis are in progress in our laboratory.

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