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Proton Permeability and the Regulation of Potassium Permeability in Mitochondria by Uncoupling Agents

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Summary. The addition of agents that uncouple electron transfer from energy conservation (uncouplers) to state 4 mitochondria causes the following ion movements: K^+ is extruded from the mitochondria in association with phosphate and possibly other anions, but not H^+. Endogenous Ca^{++} is extruded from the mitochondria, and H^+ moves in to counterbalance the Ca^{++} movement; some phosphate movement may be associated with Ca^{++} extrusion. The rate and extent of K^+ extrusion induced by uncoupler is dependent on the concentrations of external phosphate and divalent ions. Phosphate induces K^+ extrusion, while Mg^{++} and Mn^{++} inhibit it. The V_{max} of K^+ transport is 300 μmoles K^+/g protein per min. The K_m for FCCP-induced potassium extrusion is 0.25 μM at pH 7.4. The inhibitory effect of Mg^{++} is noncompetitive with respect to uncoupler concentration, but competitive with respect to phosphate concentration. The experimental evidence does not support the existence of high H^+ permeability in the presence of uncoupler. A correlation is observed between the rate of K^+ extrusion and the energy reserves supplied from the high energy intermediate. The action of uncoupler in inducing K^+ permeability is considered to arise through its action in depleting the energy reserves of mitochondria rather than through a specific activating effect of permeability by the uncoupler itself. The relationship of membrane potential to regulation of K^+ permeability is discussed.

A variety of organic compounds which contain readily dissociable hydrogen atoms and which have elaborate delocalized π electron orbitals (Szent-Gyorgyi, 1957) have shown a propensity to uncouple the electron transfer in mitochondria from the energy conservation mechanism normally leading to ATP synthesis or ion translocation. These include nitrated (e.g., DNP)[1], halogenated (e.g., PCP), and oxygenated (e.g., dicoumarol) phenols as well as derivatives of carbonylcyanidephenylhydrazone. The importance of these reagents to the study of mitochondrial metabolism arises in large part through the prospect of understanding

1 The abbreviations used are: DNP, 2,4-dinitrophenol; PCP, pentachlorophenol; FCCP, *p*-trifluoromethoxy (carbonyl cyanide) phenylhydrazone; ClCCP, m-chloro (carbonyl cyanide) phenylhydrazone; TFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; and TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

more fully the mode of normal coupling of electron transport to the energy conservation mechanism by analyzing the effects of uncoupling energy conservation. The ability of these reagents to uncouple respiration is accompanied by a variety of other effects on the energy-linked functions of these particles. Hemker (1963a, b) has reported that uncoupling agents at concentrations above the optimum for uncoupling respiration cause an inhibition of respiration and of ATPase. In kinetic experiments on the action of uncouplers, Wilson and Merz (1967) have shown that DNP, dicoumarol, and FCCP are all competitive with respect to succinate for inhibition of respiration. On the other hand, this secondary inhibition appears to show different kinetic responses for the different uncouplers.

Recently, attention has been focused upon the effects of uncouplers on the movements of ions across the mitochondrial membrane. The hypothesis of Mitchell (1961, 1966) predicts that energy conservation between electron transport and ATP synthesis is mediated through the generation of a combined pH gradient and electrical potential across the mitochondrial membrane. A corollary of this hypothesis predicts that the action of uncouplers is to give rise to a non-energy-linked conduction of protons across the membrane which would, therefore, bypass the energy-linked hydrogen movement (Mitchell, 1966). Support for this proposition has been derived from study of conductivity of artificial lipid bilayer membranes. Bielawski, Thompson, and Lehninger (1966) have reported an increase in conductivity across the bilayer induced by DNP; they consider this to be caused by the action of the dissociated and undissociated forms of DNP as a mobile carrier for protons across the membrane. These findings have been extended by Skulachev, Sharaf, and Liberman (1967) to include FCCP, ClCCP, TFB, and dicoumarol. Hopfer, Lehninger, and Thompson (1968) have shown that DNP produces a membrane potential in lipid bilayers if a pH gradient exists across the membrane. Bhowmik and Rosenberg (1968) suggest that DNP combines with the lipid to give donor-acceptor complexes.

Mitchell and Moyle (1967) showed that mitochondria in the presence of FCCP give a rapid relaxation of proton transport initiated by a pH perturbation in the external medium, but this effect is appreciable only if valinomycin is also present to increase K^+ permeability. This experimental technique gives a measure of the rate of coupled ion movements, but not a direct measure of permeability of a particular ion. The significance of this result has been placed in doubt by the findings of Rossi, Siliprandi, Carafoli, Bielawski, and Lehninger (1967) who show that hydrogen transport can be associated with the extrusion of endogenous calcium.

The transport of H^+ across the membrane on treatment with uncouplers is accompanied by the movement of other ions. Judah, McLean, Ahmed, and Christie (1965) first observed the release of endogenous K^+ on addition of DNP. At low pH, the K^+ extrusion was also accompanied by uptake of H^+. These authors also reported that this K^+ movement was inhibited by Mg^{++} and ATP. Kimmich and Rasmussen (1967) also obtained K^+ extrusion on addition of DNP and found that the rate of extrusion was higher when phosphate was present in the medium. Carafoli and Rossi (1967) observed a spontaneous K^+ extrusion on treatment with DNP only at low pH and reported that this was associated with a stoichiometric uptake of H^+. In a preliminary communication, Caswell and Pressman (1968*a*) reported that mitochondria extruded appreciable quantities of K^+ on addition of FCCP only if phosphate was present in the external medium, that the rate and also the extent of K^+ release was determined by the phosphate concentration, and that the extent of H^+ movement did not correlate with the extent of K^+ extrusion; i.e., the extruded K^+ was accompanied by very little proton uptake. Moreover, the effect of the phosphate on the K^+ extrusion and on the metabolism of the mitochondria was dependent on whether the phosphate was added before or after the uncoupling agent.

K^+ movement of a different character may be observed if valinomycin is added prior to uncoupler. Moore and Pressman (1964), Harris, van Dam, and Pressman (1967), and Kimmich and Rasmussen (1967) observed that uncouplers reversed the energy-linked K^+ uptake caused by valinomycin. Pressman, Harris, Jagger, and Johnson (1967) and Caswell (1968) showed that, whereas uncoupler alone caused only partial extrusion of K^+ from the mitochondria, the combination of uncoupler and valinomycin caused the same extensive K^+ output as did nigericin, and that uncoupler did not influence the K^+ movement caused by nigericin. The implication is that either nigericin or valinomycin plus uncoupler gave rise to rapid equilibration of the K^+, anion, and H^+ gradients. Carafoli and Rossi (1967) reported that at pH 8.0, valinomycin was unable to cause any K^+ movement (paradoxically since their traces show both a slight K^+ and H^+ movement) unless DNP was also added. The rationale proposed for this effect was that valinomycin gave rise to K^+ permeability and that uncoupler increased H^+ permeability, and only when both ions could be transported was any movement observed, in accord with the predictions of Mitchell (1966). However, the slight movement of potassium in the presence of valinomycin alone could be more readily explained if one assumes that movement represents the attainment of an equilibrium

position of energy-linked ion transport and that DNP alters this equilibrium by altering the energy sources rather than effecting a permeability increase.

In this paper, the K^+ movement induced by uncoupler alone is examined in greater detail, the range of cation and anion movements is examined, and the metabolic conditions necessary to induce K^+ release are discussed.

Methods

Rat liver mitochondria were prepared by the method of Schneider (1948) in a medium containing 0.25 M sucrose and 0.2 mM Tris ethylenediaminetetraacetate (EDTA), and then washed three times in pure sucrose.

Recordings of K^+, H^+, Ca^{++}, O_2, cytochrome *c* redox potential, light scattering, and pyridine nucleotide fluorescence were carried out in a multichannel apparatus having a single, common, calomel reference electrode and specific ion electrodes where appropriate (see Pressman, 1967). The cytochrome *c* redox potential was monitored using a vibrating platinum electrode, which also served to stir the solution, used in conjunction with the common calomel reference electrode. TMPD was added to mediate electrons between cytochrome *c* and the electrode as described by Caswell and Pressman (1968*b*). The calcium electrode was an Orion Model 92-20. The oxygen electrode was a Teflon-membrane-coated Clark electrode.

Phosphate in the medium was assayed by the method of Wahler and Wollenberger (1958) after the mitochondria were separated by sedimentation for 2 min in a Coleman Model 6-811 microcentrifuge.

Results

Caswell and Pressman (1968*a*) have previously reported that the extent of K^+ extrusion on addition of uncoupler is dependent on the external phosphate concentration. This is illustrated in Fig. 1 where the K^+ release and hydrogen uptake are shown at various concentrations of external phosphate. A slow leakage of K^+ into the potassium-free medium is observed in mitochondria respiring under state 4 conditions. The addition of uncoupler markedly increases the rate of extrusion which then falls until it reverts to its initial rate. When phosphate is omitted from the external medium, this accelerated K^+ movement on addition of uncoupler is barely discernible. In media containing phosphate, the extent of extrusion is dependent on the phosphate concentration. The pH traces show that uncoupler also initiates a movement of protons into the mitochondria. However, in marked contradistinction to the K^+ movements, the H^+ uptake is independent of the phosphate level in the medium. This contrasts with other conditions which promote K^+ movement across the mitochondrial membrane; K^+ movement induced by valinomycin (Moore & Pressman, 1964), by nigericin (Pressman et al., 1967), and by incubation

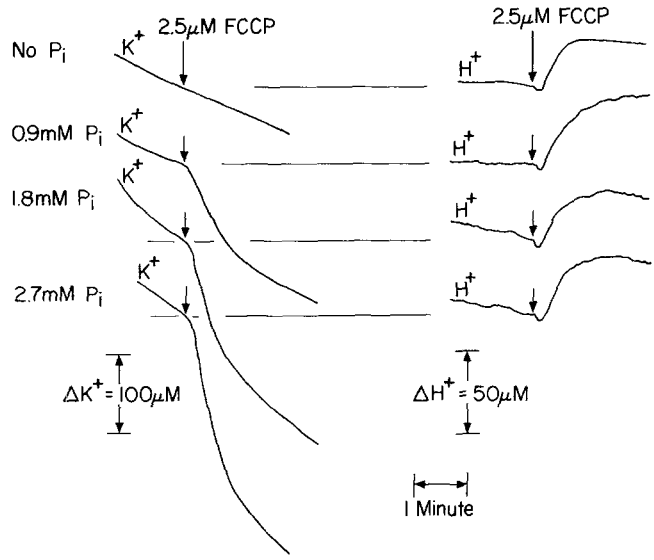

Fig. 1. Potassium and hydrogen movements on addition of FCCP. The incubation medium consists of 1.2 mM Tris glutamate; 1.2 mM Tris malate; 250 mM sucrose; mitochondria 2 mg protein/ml; and Tris phosphate where indicated. Final pH is 7.4 and temperature 22 °C. The potassium and hydrogen traces are simultaneous recordings. The traces have been normalized to show constant sensitivities. A downward deflection of either trace indicates an increase in the external medium

of the mitochondria at 37 °C (Christie, Ahmed, McLean, & Judah, 1965) are all accompanied by H^+ countermovements at reasonably constant H^+/K^+ ratios which may approach unity. Caswell and Pressman (1968*a*) have shown that on cessation of these movements the addition of nigericin causes both K^+ and H^+ movements, which indicates that the failure of H^+ to accompany K^+ extrusion in the presence of uncoupler alone is not because of the equilibrium between K^+, H^+, and anion gradients being unfavorable for H^+ transport.

As the concentration of exogenous phosphate is increased (Fig. 1), the specific extent of uncoupler-induced K^+ extrusion increases. Thus, the cessation of K^+ movement cannot be ascribed to the attainment of equilibrium between the internal and external K^+ concentrations. Moreover, under the appropriate conditions of moderate concentration of uncoupling agent and high external phosphate concentration, the amount of K^+ extruded can equal but not exceed that obtained with nigericin alone or with uncoupler plus valinomycin. It thus appears that the compartment of K^+ to which uncoupler gives access is identical to that which nigericin or valinomycin influences. The cessation of K^+ extrusion after the initial increased K^+ transport induced by FCCP must therefore

be associated specifically with a decreased permeability either of K^+ or of its accompanying gegenion.

In the presence of a low concentration of uncoupler, the rate of K^+ release is lower and the cessation of movement is not observed at all until the internal K^+ has leaked out to establish a Donnan equilibrium. Thus, the K^+ permeability increase induced by uncoupler is only transient if the uncoupler concentration is high.

The failure of correspondence between K^+ and H^+ movements raised the question of what counterions move in association with K^+ and H^+. Rossi et al. (1967) have shown that the endogenous Ca^{++} of normally prepared mitochondria is extruded on anaerobiosis. Therefore, it appeared reasonable to associate the pH change caused by uncoupler with the extrusion of endogenous Ca^{++}. This would account for the invariance of the H^+ movement despite the variable K^+ extrusion. An alternative proposition was that uncouplers induced the accumulation of phosphate or another anion so as to accompany the H^+ uptake. These hypotheses were tested using a calcium electrode as a detector of free exogenous calcium. The advantages of the Ca^{++} electrode over other analytical techniques such as atomic absorption spectroscopy are that the electrode gives continuous traces of the Ca^{++} movement and has a limit of sensitivity of approximately 10^{-6} M, which is lower than that of other techniques. The results are illustrated in Fig. 2. The addition of mitochondria causes a slight alkalinization of the medium and a reduction of

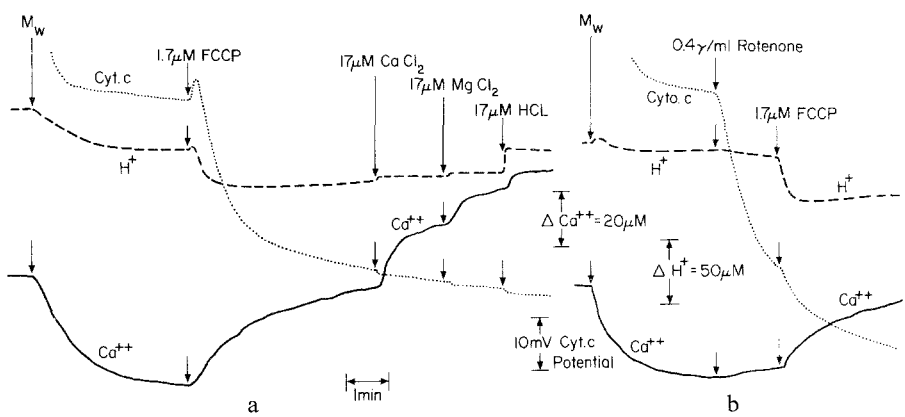

Fig. 2. Multichannel recordings to show calcium movements on addition of uncoupler. The incubation medium consists of 1.2 mM Tris glutamate; 1.2 mM Tris malate; 250 mM sucrose; and 13 μM TMPD. Final pH is 7.4 and temperature 22 °C. Additions to the medium are: mitochondria 2 mg protein/ml, and further reagents where indicated. An upward deflection of the pH and pCa traces indicates increase in the external medium. A downward deflection of the cytochrome *c* trace indicates oxidation

Ca^{++} activity. This change in the Ca^{++} trace could represent an interaction of the ion exchange interface of the electrode with the mitochondria. The addition of rotenone in Fig. 2b gives rise to oxidation of cytochrome *c* and also to the slow extrusion of Ca^{++}; a slow alkalinization corresponding to the release of Ca^{++} is also detectable. The addition of FCCP at a concentration sufficient to give rise to extensive oxidation of cytochrome *c* either in the presence or in the absence of rotenone promotes rapid extrusion of Ca^{++}; at the same time, the pH trace, after an initial small and characteristic acidification, indicates a substantial H^+ uptake. These experiments have been carried out in a medium free of added phosphate where K^+ extrusion on addition of uncoupler is negligible, but a similar release of Ca^{++} is observed if phosphate is present. The further calibration of the traces with additions of Ca^{++}, Mg^{++}, and HCl standards is shown. These indicate that the electrode has some response to Mg^{++} as well as to Ca^{++}. In practice, the electrode is primarily an indiscriminate indicator of divalent ions at these low concentrations. However, the movement of Mg^{++} under the influence of metabolic changes has been reported by Carafoli, Rossi, and Lehninger (1964) to be slow, and so it appears most likely that Ca^{++} extrusion accounts for the bulk of the Ca^{++}-electrode changes observed here. The sensitivity of the electrode to pH changes is also a feature of the electrode at low Ca^{++} concentrations. However, the pH change when FCCP is added would appear as a reduction of the movement of the Ca^{++} trace. The Ca^{++} electrode has previously been applied to observation of Ca^{++} uptake into mitochondria by Chance and Yoshioka (1966) and into microsomes by Johnson and Pressman (1968). These workers have reported a response time to Ca^{++} addition of approximately 10 sec. This slow response is also observed in this experiment which accounts for the failure of closer correlation of the pH and pCa traces with respect to time. The Ca^{++} trace has also been calibrated in a separate experiment to determine its linearity over the range of Ca^{++} change shown in the figure. The slight drift of the Ca^{++} trace subsequent to Ca^{++} extrusion precludes an exact comparison of the H^+ and Ca^{++} movements, but the H^+/Ca^{++} ratio is estimated as approximately 3:2.

The Ca^{++} extrusion demonstrated in Fig. 2 supplies a counterion for the observed H^+ uptake. However, the counterion associated with K^+ extrusion is still indeterminate. Gamble and Hess (1966) reported a high inorganic phosphate content in mitochondria. It appeared likely that phosphate was extruded in association with K^+. This was examined by analysis of the external medium for phosphate before and after FCCP

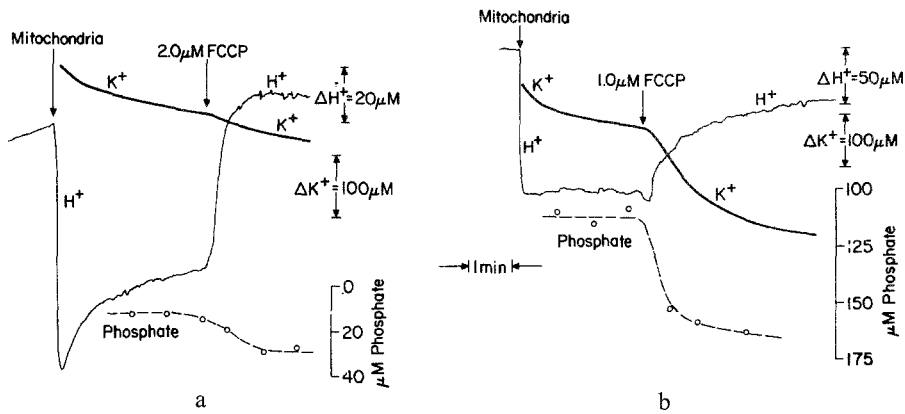

Fig. 3. Multichannel recordings to show phosphate movements on addition of uncoupler. The incubation medium consists of 1.3 mM Tris glutamate; 1.3 mM Tris malate; 250 mM sucrose; and (in Fig. 3b) 125 μM Tris phosphate. Final pH is 7.4 and temperature 22 °C. Additions to the medium are mitochondria 3 mg protein/ml and FCCP where indicated. A downward deflection of the traces indicates an increase in the external medium. Aliquots (0.5 ml) of suspension are withdrawn from the medium for phosphate assay and centrifugation begun at the time indicated by the points. The centrifugation was continued for 2 min and the supernatant was withdrawn and analysed for inorganic phosphate

addition. In Fig. 3, the phosphate analysis is shown for the situation where very little K^+ release occurs in the absence of added phosphate (Fig. 3a) and where a substantial K^+ release is observed in the presence of 100 μM phosphate added (Fig. 3b). Fig. 3a shows that addition of FCCP caused 62 μM H^+ uptake and approximately 10 μM K^+ egress, whereas an increase in external phosphate of approximately 15 μM was observed. It appears, therefore, that some phosphate may accompany Ca^{++} extrusion. In Fig. 3b, there is a substantial extrusion of phosphate accompanying K^+ release. Since the mitochondria are centrifuged for 2 min in order to separate the supernatant from the mitochondria, the kinetics of the phosphate release may not be accurately represented by the time scale for the phosphate analyses, which refer to the time at which the centrifugation was initiated. The continuous K^+ extrusion renders quantitative estimates of the K^+: Pi ratio difficult to determine. A value of approximately 3:1 is estimated from the figure. The charge of the phosphate will be ca. −1.5 at the pH of the medium, and therefore the phosphate release does not account for the full K^+ release; it seems likely that other anions accompany the output of K^+ and these could include the substrates glutamate and malate or any of the other numerous anions that exist in the mitochondria.

Both Mg^{++} and Mn^{++} cause inhibition of the uncoupler-induced K^+ efflux. However, neither has any effect on the extent or time course of

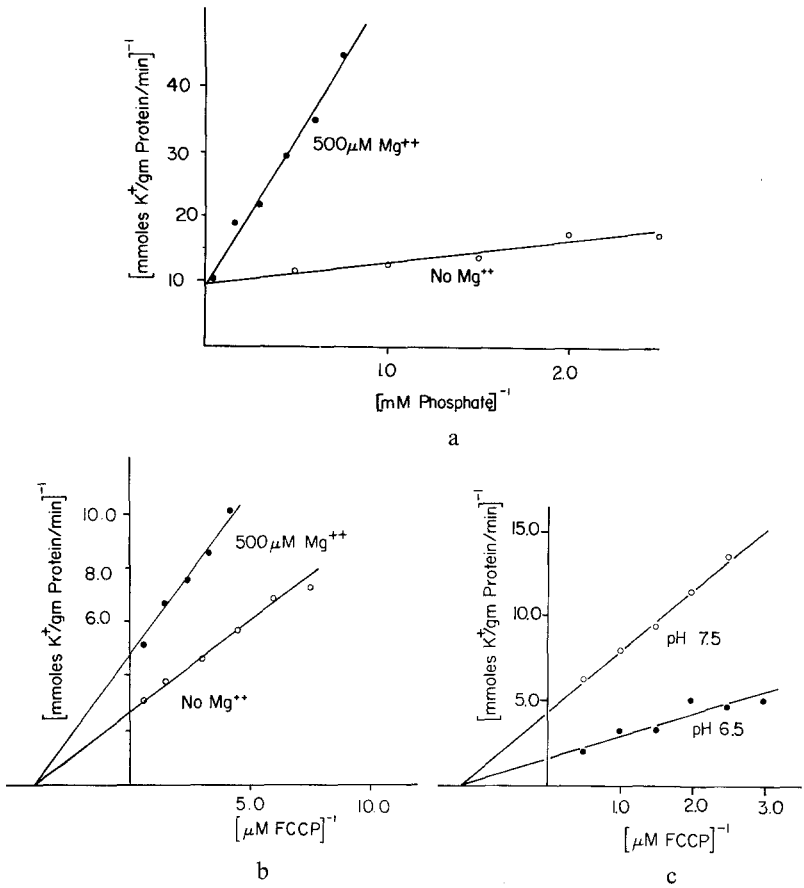

Fig. 4. Lineweaver-Burke plots of the rate of potassium extrusion at varying phosphate and uncoupler concentrations. The incubation medium consists of 2.5 mM Tris glutamate; 2.5 mM Tris malate; 300 mM sucrose; in Fig. 4a, 4.0 mM Tris Cl; and in Fig. 4b and c, 2.0 mM Tris phosphate. Unless otherwise stated, the pH is 7.4 and temperature 22 °C. In Fig. 4a, the potassium extrusion was initiated by addition of 2.0 μM FCCP

onset of inhibition of movement that follows the activation when high concentrations of uncoupler are present. In this respect, inhibitory properties of the divalent ions resemble the activating properties of phosphate. The kinetic characteristics of these interactions are illustrated in Fig. 4 where Lineweaver-Burke plots are shown comparing Mg^{++} with phosphate and Mg^{++} with uncoupler. The traces show good approximation to straight lines over the range of uncoupler and phosphate concentrations examined. Since the mechanism of induction of K^+ transport is obscure, the justification for applying Michaelis-Menten kinetics to analysis of the effect of different reagents on the rate of ion transport must rest solely on the empirical fit of the data to the theoretical

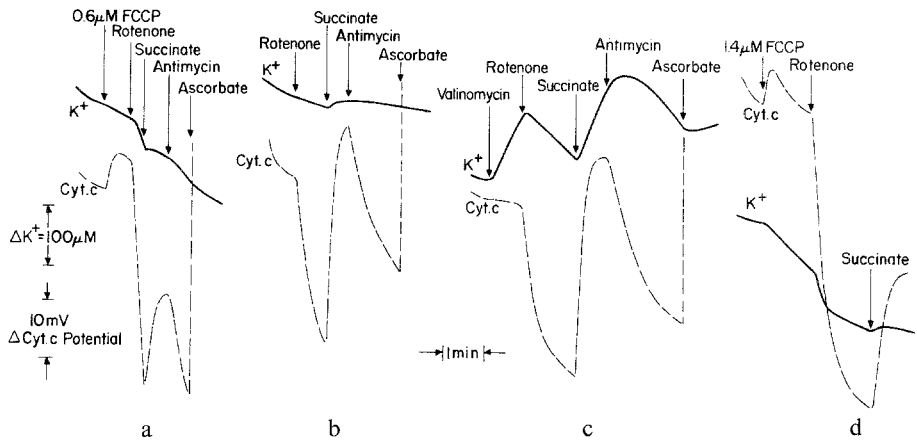

Fig. 5. Potassium and cytochrome *c* redox potential traces showing ion permeability and transport properties induced by FCCP and calinomycin. The incubation medium consists of 3 mM Tris glutamate; 3 mM Tris malate; 2.5 mM Tris phosphate; 300 mM sucrose; 13 μM TMPD; and mitochondria 2 mg protein/ml. Final pH is 7.4 and temperature 22 °C. Additions to the suspension are FCCP as indicated; 0.7 μg/ml rotenone; 2 mM Tris succinate; 0.07 μg/ml antimycin A; 0.7 mM Tris ascorbate; and 0.035 μg/ml valinomycin. A downward deflection in the potassium trace indicates increase in the external medium. Oxidation of cytochrome *c* is indicated by a downward deflection

curve. The straight lines of Fig. 4 show adequate confirmation of the applicability of Michaelis-Menten kinetics in this study. The traces show that inhibition of K^+ movement by Mg^{++} is competitive with respect to phosphate activation and noncompetitive with respect to FCCP concentration. Fig. 4b shows that the apparent K_m for FCCP at an external pH of 7.4 is 0.25 μM. Fig. 4c shows that FCCP concentration is noncompetitive with respect to pH changes. According to the view that FCCP exerts its action by causing transport of protons across the mitochondrial membrane (Mitchell, 1966), one might expect to obtain a competitive interaction between pH and uncoupler concentration. However, a noncompetitive interrelation does not unequivocally indicate that the proposal is inaccurate, since other factors in the system may be influenced nonspecifically by altering the pH, thereby affecting the observed data.

In Fig. 5, a comparison is made of the influence of inhibitors and substrates on the rate of K^+ movement induced either by uncoupling agents or by valinomycin. In Fig. 5a, the addition of a low concentration of FCCP to mitochondria respiring on glutamate and malate is insufficient to give rise to a detectable increase in K^+ efflux (phosphate present). The addition of rotenone now induces an immediate stimulation of K^+ egress and oxidation of cytochrome *c*. The addition of succinate causes the cessation of K^+ efflux, but not the reversal of movement. The subsequent

addition of antimycin A leads to a stimulation of K^+ efflux at a rate substantially lower than that induced by rotenone. The lesser stimulation of K^+ movement included by antimycin as opposed to rotenone results from the slight bypass of antimycin inhibition permitted by the TMPD present so that antimycin acts less effectively as a respiratory inhibitor (Lee, Nordenbrand, & Ernster, 1964). Addition of ascorbate then reduces the antimycin-induced K^+ leakage. It is seen, therefore, that respiratory inhibitors stimulate K^+ leakage whereas substrates inhibit the release. On the other hand, if no FCCP is added to the mitochondrial medium, then no increase in K^+ flux is evidenced on addition of the inhibitors rotenone and antimycin (Fig. 5b). The change in the K^+ trace on addition of succinate is an electrode artifact caused by the marked change in electrolyte concentration. A comparison of the effects of valinomycin is shown in Fig. 5c where the antibiotic is added to respiring mitochondria; in this case, the response of K^+ movement to inhibitors or substrates is altogether different from that observed in the presence of FCCP. In Fig. 5c, valinomycin induces K^+ uptake consonant with utilization of energy from oxidation of glutamate-malate. The addition of inhibitors causes K^+ extrusion, but addition of substrates initiates K^+ uptake. The responses in the presence of valinomycin are in accord with the proposal that valinomycin induces an energy-linked K^+ transport and that changes in the rate or direction of K^+ movement are to be associated with changes in the driving forces rather than in the permeability of the mitochondria to K^+ (Moore & Pressman, 1964). The responses to inhibitors in the presence of FCCP are, on the other hand, of a different character, since no reversal of K^+ efflux is observed under any conditions studied. It will be argued in the discussion that the evidence favors the view that uncouplers and inhibitors are exerting complementary effects primarily on K^+ permeability and only secondarily on the forces that determine the direction of movement. The responses to inhibitors in the presence of FCCP are consonant with the view that the K^+ permeability, but not necessarily the equilibrium K^+ gradient, is under metabolic control. The presence of oligomycin does not influence the K^+ movements. It therefore appears that the K^+ permeability is determined by the level of high energy intermediate in the mitochondria. Fig. 5d shows that if 1.4 μM FCCP is added to the mitochondrial suspension, then a slow egress of K^+ occurs. If rotenone is added subsequently, the egress is rapid but of short duration. It appears, therefore, that not only is the induction of K^+ transport by uncouplers under metabolic control, but so also is the inhibition of K^+ transport by higher levels of uncoupler.

The observation that addition of uncoupling agents causes extensive swelling of the mitochondria in the presence of phosphate (Chappell & Crofts, 1966; Azzi & Azzone, 1965) prompted an examination to determine if a correlation existed between volume changes in mitochondria and K^+ permeation. The results are illustrated in Fig. 6 where the back scatter of light from the suspension, external K^+ concentration, and pyridine nucleotide fluorescence are monitored on addition of FCCP under a variety of conditions. In Fig. 6a, the incubation medium contains phosphate, and the traces show that addition of uncoupler in concentrations sufficient to cause an increase in K^+ egress also causes a substantial reduction in light-scattering signal indicative of swelling of the mitochondria. There is a delay in the onset of swelling after uncoupler addition so that the correlation of swelling with K^+ output does not apply strictly with respect to time, but the overall swelling is of sufficiently large amplitude that the light-scattering signal approaches close to zero. This is presumably commensurate with extensive damage to the outer membrane and possibly to the inner membrane. However, by the time the maximum swelling has been attained, the K^+ permeability has become inhibited argues in favor of the intactness of the inner membrane even under these severely swollen conditions. In Fig. 6b, the medium is identical, and addition of FCCP in low concentration such that little K^+ efflux occurs does not materially affect the light-scattering trace. If the K^+ efflux is now induced by the addition of rotenone instead of further FCCP, then, in contrast to the results in Fig. 6a, there is a contraction of the mitochondria, presumably as a response to the alteration in osmotic pressure in the mitochondria, and the contraction is succeeded by inhibition of the slow swelling which occurred prior to any additions. The contraction of the mitochondria along with a further K^+ loss upon subsequent addition of nigericin shows that K^+ permeability had become inhibited prior to the establishment of gradient equilibrium. If the medium is devoid of added phosphate (Fig. 6c), then addition of uncoupler, although it causes oxidation of the endogenous nicotinamide adenine dinucleotide (NAD) similar to that observed in a medium containing phosphate, has very slight effect on either the light scattering or the K^+ extrusion. Finally (Fig. 6d), Mg^{++} is supplemented to a medium containing phosphate. The rate of K^+ movement on addition of uncoupling agent is substantially lower than in the absence of Mg^{++}, but the uncoupler-induced swelling is completely abolished by Mg^{++}. During the course of the reaction, when the medium becomes anaerobic as indicated by the reduction of NAD, an increased K^+ egress and contraction of the mitochondria occur.

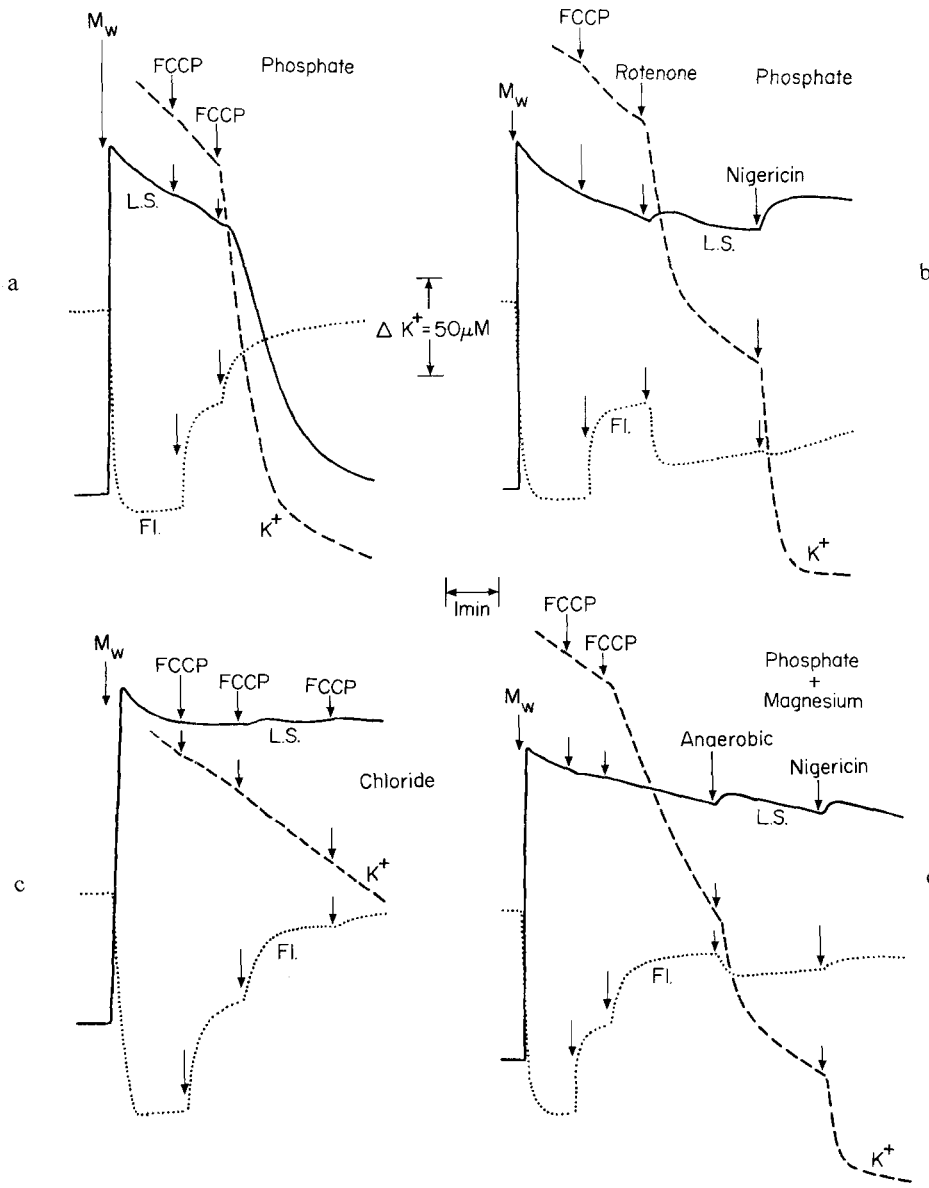

Fig. 6. Volume changes associated with potassium extrusion initiated by uncoupler. The incubation medium consists of 6 mM Tris glutamate; 6 mM Tris malate; 250 mM sucrose; and where indicated 5 mM Tris phosphate; 10 mM Tris Cl, and 2.5 mM $MgCl_2$. Additions to the medium are: mitochondria 2 mg protein/ml; 1.0 μM FCCP; 1.0 μg/ml rotenone; and 0.02 μg/ml nigericin. A downward deflection of the trace indicates increased external potassium (K^+), decreased light scattering (*L.S.*), and increased fluorescence (*Fl.*)

This increased K^+ egress caused by anaerobiosis is presumably similar to that induced by rotenone. Subsequent addition of nigericin shows that the K^+ extrusion had almost, but not quite, reached the equilibrium point.

Whereas a superficial correlation between light-scattering changes and K^+ efflux is evident from the similar effects of phosphate and Mg^{++} on both parameters, a quantitative correlation has not been observed between the volume changes and any of the functions of K^+ movement (i.e., the rate of extrusion, the extent of extrusion, or the onset of inhibition of extrusion). Although the light-scattering determinations may not correlate strictly with the volume of the mitochondria, the parameter gives a close correspondence to volume changes and, in any event, has served as the criterion of volume changes of mitochondria under a variety of conditions (Chappell & Crofts, 1966; Azzi & Azzone, 1965). The range, variety, and mystifying complexity of volume changes caused by different reagents (Chappell & Crofts, 1966; Azzone & Azzi, 1966) render interpretation of the volume change induced by uncouplers difficult. A unified explanation for the light-scattering changes observed and their failure to correlate precisely with K^+ movement is not readily discernible. It is, however, quite apparent that the explanation for the K^+ permeability changes induced by uncoupler should not be sought in nonspecific damage to the mitochondrial membrane.

Discussion

Criteria of Permeability

In later sections of this paper, the alteration and regulation of mitochondrial K^+ permeability will be discussed. However, in order to examine this topic, an understanding of the meaning of the term permeability is a prerequisite. Traditionally, permeability may be determined by radioisotope exchange where the permeant ion is in equilibrium or in a steady state of flux across the membrane, or it may be determined by applying Fick's law to estimate the relationship between flow and force. In the study of ion permeability, the problem is complicated by the dual forces that are acting on the ion, the diffusion potential, and the membrane potential. This situation has been analyzed by Goldman (1943) in the constant field equation. Recently, attention has been focused on the limitations of equations that assume a linear relationship between force and flow when applied to carrier-mediated or energy-linked transport. Rosenberg and Wilbrandt (1963) and Katchalsky and Spangler (1968) have developed equations to define the parameters of carrier-mediated transport. However, these equations have not been extended to encompass the movement of charged species under the influence of a membrane potential. There is no evidence in mitochondria to determine whether the intrinsic K^+

permeability is to be associated with diffusional movement through a channel or carrier-mediated transport. In the latter circumstance, the rate of K^+ transport would be expected to exhibit saturation with respect to K^+ concentration, K^+ gradient, and membrane potential.

In the case of mitochondrial K^+ transport, the situation is further complicated by the possibility that the K^+ movement is energy-linked. K^+ movement induced by incubating the mitochondria at 37 °C (Christie et al., 1965) or by adding valinomycin (Moore & Pressman, 1964) is linked to electron transport. Thus, three forces may determine the magnitude and direction of K^+ flow: the membrane potential, the diffusion potential, and the potential from the energy-linked reaction. In the discussion that follows, permeability changes are characterized with reference to the three types of force acting on ion movement and the observed flow which results from these forces. In this way, a distinction is made between changes in the extrinsic properties of the system and alterations of permeability that represent the intrinsic properties of the membrane.

Evidence for Permeability Change Induced by Uncoupler

A mode of K^+ transport across the mitochondrial membrane is described in this paper that differs radically from previously described ion transport. It is appropriate at this stage to differentiate these various modes of transport and to describe their salient characteristics. We therefore distinguish K^+ transport as arising in four ways:

1. Spontaneous K^+ transport at a low rate in fresh mitochondria (Share, 1958) or at an accelerated rate in mitochondria treated to increase permeability by incubation at 38 °C (Christie et al., 1965), at acid pH (Carafoli & Rossi, 1967), or by treatment with EDTA (Azzone & Azzi, 1966), parathyroid hormone (Rasmussen, Fischer, & Arnaud, 1964), or Zn^{++} (Brierley, Bhattacharyya, & Walker, 1966). These K^+ movements have not all been thoroughly characterized, but they appear to occur in association with an H^+ countermovement and anion movement and can take place against the chemical gradient through utilization of energy derived from oxidizable substrates or ATP. There may be class distinctions within this group, but there is no evidence available which distinguishes different modes of ion transport under the various induction conditions.

2. Valinomycin-induced energy-linked K^+ transport. This was first characterized by Moore and Pressman (1964), and the bulk of evidence

indicates that valinomycin transports the K^+ in the form of a clathrate complex (Pressman et al., 1967). The movement can take place against its chemical gradient through use of energy derived from electron transport or ATP, and is associated with anion transport and H^+ movement. The mechanism of energy linkage is likely to be similar to that observed with spontaneous ion movement. Other antibiotics, e.g., gramicidin or the actins, mimic the effects of valinomycin with different degrees of ion specificity.

3. Nigericin-induced K^+ transport. The effects of nigericin on K^+ transport were first observed by Graven, Estrada-O, and Lardy (1966). Pressman et al. (1967) have characterized the action of nigericin as a mobile carrier inducing a non-energy-linked exchange of K^+ for H^+, giving rise to an equilibration of K^+, H^+, and, indirectly, of anion gradient. The antibiotic appears to transport the K^+ in a neutral complex in exchange for H^+. Other antibiotics such as dianemycin mimic the effects of nigericin with certain differences in ion specificity (Lardy, Graven, & Estrada-O, 1967).

4. Potassium movement induced by uncoupling agents or by Ca^{++} transport (Judah et al., 1965; Caswell & Pressman, 1968*a*; Berger, 1957; Caswell, 1968) which represents a reversible alteration of K^+ permeability. This is distinguished from the previous types of ion movement by the apparent inability of protons to act as counterions for the K^+ and by the transient nature and reversibility of the ion permeability. It is possible that histones induce a similar increase in K^+ permeability (Johnson, Mauritzen, Starbuck, & Schwartz, 1967), although the relation between this and uncoupler- or Ca^{++}-induced K^+ movements is not clear as yet.

The evidence of Figs. 1, 2, and 3 shows that on addition of uncoupling agent the following ion movements take place. Endogenous Ca^{++} is extruded from the mitochondria and H^+ is taken up; there may be some phosphate ion movement in association with the Ca^{++} transport. The K^+ is extruded from the mitochondria in association with phosphate and possibly with other anions. The nature of Ca^{++} movement lies beyond the scope of this paper and will not be discussed further; the implications of the K^+ movement will be considered.

A prerequisite for determining permeability changes in mitochondria under the influence of reagents that alter metabolism is an understanding of the initial permeability parameters of the mitochondria. The initial metabolic condition that has been chosen for these experiments is state 4. In the absence of external K^+, under the conditions described in this

paper, the addition of ADP or of inhibitors (antimycin or rotenone) does not cause any change in the ion movements. Under these conditions, K^+ electrode traces show a slow leakage of K^+ from the mitochondria in conditions where the K^+ gradient across the mitochondria is high, implicating a low K^+ permeability. Moreover, data obtained using $^{42}K^+$ shows a low incorporation of radioactivity into the mitochondria (Harris, Catlin, & Pressman, 1967). At 10 °C and in the presence of 2.5 mM phosphate, Harris et al. (1967) showed an influx of labelled K^+ of about 0.3 μmoles K^+/g protein per min. The anion permeability, on the other hand, is complicated by distinctions between the ability of anions to accumulate (Chappell & Haarhoff, 1967) and ability of anions to penetrate the membrane in exchange studies (Harris & Manger, 1968). Chappell and Haarhoff (1967) have demonstrated high phosphate permeability although their measurements were made under very different conditions from those described here, and some doubt must exist regarding the justification of extrapolating permeability determinations under different metabolic conditions. The extent of H^+ permeability across the mitochondrial membrane is still controversial. High H^+ permeability has been demonstrated for Ca^{++} transport and valinomycin-induced K^+ transport to account for H^+ countermovements observed, but this high permeability does not necessarily apply in state 4 conditions. Mitchell and Moyle (1967) have demonstrated a low H^+ permeability in anaerobic mitochondria, but this applies only to coupled movements of H^+ and cations in which cation permeability may be rate limiting.

The K^+ electrode trace shows that addition of ADP or rotenone (*see* Fig. 5) to state 4 mitochondria does not cause an increased K^+ extrusion. The low K^+ efflux could arise either through a low permeability for K^+ or a low permeability for anion, or it could arise through the equilibration of the electrochemical potential of K^+ with energy from electron transport under conditions of high K^+ and anion permeability. However, rotenone and ADP both reduce the availability of energy sources which would change the equilibrium K^+ gradient and, if K^+ permeability were high, would thus give rise to rapid K^+ efflux until that equilibrium is established. This does not occur and so the explanation for the low K^+ efflux must lie in a permeability barrier. The addition of rotenone or ADP may change the forces which act on K^+ transport without altering the rate of flow which is a situation appropriate to the type of saturation kinetics discussed in the previous section. In view of the absence of effect of rotenone or ADP and the possible condition of saturation kinetics, the rapid efflux of K^+ induced by uncouplers must arise through a change of permeability

of K^+ or its counterion. Moreover, Harris et al. (1967) have shown a state 4 steady state flux of $^{42}K^+$ of about 0.3 μmole K^+/g protein per min. Unidirectional K^+ efflux in these experiments is about 10 μmoles K^+/g protein per min in state 4, whereas the maximum rate of flow of K^+ from electrode traces in the presence of uncoupler is shown from Fig. 4 to be about 300 μmoles K^+/g protein per min, which represents a very substantial change specifically in K^+ permeability.

The cessation of K^+ movement when high concentrations of uncoupler are used must also result specifically from a change in membrane permeability. At high uncoupler concentrations, energy sources from electron transport or ATP are depleted; hence, the K^+ gradient will tend towards a minimum where the electrochemical gradient is zero. However, K^+ transport in the presence of high uncoupler concentrations normally ceases or reverts to its state 4 rate before the maximum amount of K^+ is extruded, as shown from the subsequent addition of nigericin. This cessation of K^+ movement could result from a change in K^+ permeability or in anion permeability. There is some evidence to support the view that it is specifically K^+ permeability which is reduced. Harris et al. (1967) quote a very low permeability of $^{42}K^+$ after addition of uncoupler; it should, however, be emphasized that this experiment was carried out in the absence of added phosphate. Further suggestive evidence to support the view that K^+ transport is specifically inhibited is the fact that K^+ extrusion reverts to its original state 4 rate rather than dropping to zero; this implies that the original permeability change has been reversed. Nevertheless, it remains possible that it is the anion movement which is inhibited.

Evidence bearing on the role of uncoupler in inducing K^+ permeability changes is provided by Fig. 5. K^+ extrusion may be induced by low concentrations of uncoupler in concert with rotenone or antimycin, or it may be induced by higher concentrations of uncoupler alone added to state 4 mitochondria. This synergistic action of uncoupler and inhibitor suggests that K^+ permeability changes should not be associated with a specific transport of K^+ by the uncoupler itself. This is confirmed by the fact that the increase in potassium permeability may be induced by calcium in place of uncoupler (Caswell, 1968) which does not favor a role of uncoupler as a transporting agent. The increase in K^+ permeability caused by uncoupler is a reversible phenomenon as shown in Fig. 5. This reversibility is even more markedly demonstrated with Ca^{++} where the completion of Ca^{++} uptake is accompanied by cessation of K^+ movement (Caswell, 1968). It follows, therefore, that the phenomenon of induction of K^+ permeability is nonspecific regarding the active inducing agent but

is specific for the metabolic conditions of the mitochondria. The feature that is common to all the conditions giving rise to high permeability (high uncoupler, low uncoupler plus rotenone, or Ca^{++}) is that these reagents cause severe depletion of the energy resources of the mitochondria. This feature supports the conclusion that it is the action of reducing the energy level of high energy intermediate that is responsible for the increase in K^+ permeability and not a specific ion transport-inducing activity intrinsic to the reagent itself. Accordingly, this represents the first experimental support for a natural mechanism of metabolic control of ion permeability in mitochondria. The data of Fig. 5 give substantial support to this conclusion; the additions of inhibitors in the presence of low concentration of FCCP caused activation of K^+ efflux whereas the addition of substrate caused inhibition.

The contrast between the K^+ traces of Fig. 5a, b, and d with that of 5c where valinomycin replaces uncoupler is marked. In the presence of valinomycin, permeability is high and the addition of inhibitors and substrates affects the direction of energy-linked K^+ transport. In the presence of uncoupler, the effects of inhibitors and substrates are manifested quite differently, since K^+ movement is invariably in one direction and changes in the rate of K^+ movement reflect changes in permeability rather than in the equilibrium K^+ gradient, which is, under the experimental conditions described, favorable for discharge of K^+.

The energy state of mitochondria may be defined in terms of the free energy of breakdown of high energy intermediate. This will be referred to as the chemical potential of $\sim$. This phrase is not intended to imply any particular hypothesis of coupling between ion and electron transport. When uncoupler is added to state 4 mitochondria, a competitive interaction exists between substrate which is supplying energy to maintain the $\sim$ potential and uncoupler which is discharging it. If, however, rotenone is added, the source of metabolic energy is eliminated; the uncoupler will effect a discharge of $\sim$ and an energy-dependent K^+ permeability increase will occur. If higher concentrations of uncoupler are added (Fig. 5d), then K^+ permeability is increased even when added to state 4 mitochondria; the addition of rotenone causes a further increase of permeability, although of short duration. A doubt might arise as to the validity of the mechanism proposed above in view of the inadequacy of rotenone or ADP by themselves in reducing permeability changes, since both these reagents also reduce the $\sim$ potential. However, the difference between rotenone or ADP and uncoupler is a quantitative one since neither of the former reagents is able to effect such a severe depletion of energy. Hence, the $\sim$

potential is above the threshold for inducing a permeability increase. Furthermore, it is not known whether the K^+ extrusion induced by uncoupler is energy-linked. If the K^+ movement is energy-linked, then any trend toward extrusion of K^+ on addition of rotenone would tend to cause an increase in $\sim$ potential and so counteract the permeability increase.

The high K^+ permeability may be sustained under the right conditions where the $\sim$ potential has been appropriately reduced by uncoupler. A cessation of K^+ movement may occur if the potential rises; in this instance, it is reasonable to expect that a simple reversal of the initial K^+ permeability is responsible. On the other hand, under conditions where the $\sim$ potential drops to a very low level due to the addition of a high level of uncoupler (Fig. 1) or to the addition of inhibitors of respiration together with uncoupler (Fig. 5d), the K^+ movement also ceases. Unlike the onset of K^+ permeability, the cessation at high uncoupler concentrations is independent of the presence of phosphate or Mg^{++} and appears to be controlled entirely by the $\sim$ potential.

Thus, there is evidence that within a restricted range of $\sim$ potential there exists a high K^+ permeability, and that beyond these values both at higher and at very low levels of $\sim$ potential there exists a restricted permeability to K^+.

The question arises as to the manner in which the $\sim$ potential regulates the ion permeability. Many possible interpretations are available. However, this phenomenon of permeability control is by no means restricted to mitochondria and has been accepted for many years as a feature of transmission of nerve impulses (Hodgkin & Huxley, 1952). Recently, this control phenomenon has been reproduced in artificial lipid bilayers by adding a factor (excitability-inducing material) which displays a permeability to cations that is controlled by the membrane potential (Mueller & Rudin, 1968). It appears reasonable, therefore, to relate control of K^+ permeability in mitochondria to the physical gating phenomena induced by electrical potential or possibly to allosteric changes in carriers or intermediates induced by changes in chemical potential. If the K^+ and counterion movement were non-energy-linked, then the membrane potential would be determined by Goldman's equation. Thus, if the K^+ permeability in state 4 mitochondria is low, then the membrane potential will be the anion diffusion potential and will, under most circumstances, be positive within the mitochondria. If, on the other hand, the ion movements are energy-linked, then two different considerations arise. Either the anion or the K^+ is the energy-linked ion. Considering first the possibility

that the anion is energy-linked, provided the K^+ permeability is low, the membrane potential will be determined by the following equation:

$$E_m = E_{A'} + \frac{1}{nF} \Delta F''.$$

E_m represents the membrane potential; $E_{A'}$, the anion diffusion potential, positive if $[A']$ inside is greater than $[A']$ outside; n, the number of anions transported per equivalent $\sim$ dissipated; and $\Delta F''$, the free energy of hydrolysis of high energy intermediate. Hence, the energy linkage will tend to make the inside of the mitochondria more negative and thus reduce or reverse the membrane potential. Moreover, the energy linkage will have a very marked controlling effect on the membrane potential.

If the K^+ ion is energy-linked, the situation is more complicated. If the K^+ permeability were zero, then any energy linkage would have no effect on the membrane potential which would be controlled entirely by the anion diffusion potential. If, on the other hand, as exists in state 4 mitochondria, the K^+ permeability were low but finite, then energy linkage of K^+ would have a slight control on the membrane potential. Moreover, the energy linkage would tend to make the inside of the mitochondria more positive and hence reinforce the anion diffusion potential. The situation thus arises that, if K^+ is energy-linked, then $\Delta E_{A'}$ primarily determines the membrane potential, whereas if anion is energy-linked the E_m is determined by $\Delta E_{A'}$ and $\Delta F''$.

The $\Delta E_{A'}$ is the gradient of phosphate across the membrane. Thus, it becomes possible to visualize the activating effect of phosphate on K^+ permeability as arising from the variation in membrane potential effected by varying the external phosphate concentration. The specificity for phosphate in activating K^+ permeability may well arise through the action of the phosphate carrier alone among anion carriers in inducing an electrophoretic ion transport as opposed to an exchange diffusion.

It is less easy to explain the activating effect on K^+ permeability of de-energizing the mitochondria in terms of a direct effect on membrane potential since the only scheme in which $\Delta F''$ affects E_m is that in which anion is energy-linked; in this situation, the decrease in $\Delta F''$ opposes the decrease in $\Delta E_{A'}$, both of which would be expected from the experimental evidence to increase K^+ permeability. It is, however, possible to conceive that the $\sim$ potential of the mitochondria might alter the distribution of charges within the mitochondrial membrane and thereby alter the permeability.

The Proton Permeability of Mitochondria

In the foregoing discussion, particular emphasis has been placed on the manner of K^+ transport across the mitochondrial membrane. However, the particular mode of ion transport elicited by addition of uncouplers is of considerable interest in the context of present hypotheses of the action of uncouplers and the mode of anion transport. Model membrane studies carried out by a number of workers have shown that uncoupling agents increase conductivity (Bielawski et al., 1966; Skulochev et al., 1967). Recently, Hopfer et al. (1968) have demonstrated the generation of an electrical potential across a lipid bilayer through a pH gradient when FCCP is added to the membrane. These model membrane studies have been interpreted as implicating the passage of protons across the mitochondrial membrane as a mode of action of uncouplers, in accord with the theory of Mitchell (1966). Model membrane studies have proved valuable as analogies of mitochondrial ion movements in the study of antibiotics which induce cation transport (Mueller & Rudin, 1967). However, the appropriate evidence of increased H^+ permeability in the presence of uncoupler in mitochondria has not been forthcoming. Mitchell and Moyle (1967) have demonstrated an increased H^+ permeability induced by FCCP and valinomycin, but this cannot be considered an appropriate demonstration of the effect of FCCP singly on mitochondria.

The experimental results of this paper show that uncouplers induce Ca^{++} extrusion and H^+ uptake. It is still not certain whether this H^+ movement represents a preexisting H^+ permeability or a permeability increase induced by the Ca^{++} transport, but the pH change cannot be cited as evidence of increased H^+ permeability specifically induced by uncoupler since Ca^{++} movement is invariably associated with high H^+ permeability even in the absence of further added reagents. A conspicuous feature of uncoupler-induced ion permeability changes is the extrusion of K^+ accompanied by anion, but not by any pH change under any of the circumstances that have been examined. In the presence of uncoupler where energy supplies are depleted, the equilibrium of ion gradient is represented by:

$$\Delta E_{A'} = \Delta E_{K^+} = \Delta \mathrm{pH}.$$

This represents equal diffusion potentials of the ions. In the presence of FCCP plus valinomycin or of nigericin, a K^+, H^+, and A' movement occurs to establish this equilibrium, but in the presence of FCCP or FCCP plus rotenone the only movements are those of K^+ and A'. The implication of this experimental observation is that H^+ does not traverse the membrane because there is no H^+ permeability.

The movement of cations across the mitochondrial membrane is associated normally with depletion of energy. The utility of cation transport in physiological metabolism is not fully understood. However, the movement of anions across the membrane is an important integral part of mitochondrial metabolism, since physiological substrates are themselves anions and their transport across the mitochondrial membrane is a prerequisite of oxidation. Mitchell (1966) has proposed that the energy necessary to cause accumulation of anions within the mitochondria is supplied from the pH gradient induced by chemiosmotic pumping of protons. This view has received support from Chappell and Haarhoff (1967) who propose that a neutral exchange of OH' for anion occurs to cause uptake of the anion from energy of the pH gradient. It seems appropriate at this stage to refer to the exchange diffusion carriers postulated in Mitchell's presentation of the chemiosmotic theory (1966). There is a requirement for neutral exchange carriers for all ion movements in normal respiration in order to maintain the membrane potential induced by hydrogen pumping. The existence of a K^+/H^+ exchange diffusion carrier has already been the subject of criticism (Caswell, 1968). The presence of an OH'/anion exchange diffusion requires that there be a trend toward equilibrium between the anion and pH gradients. After the addition of uncoupling agent, however, there occurs a K^+ and anion movement altering the anion gradient substantially, but there is no concomitant H^+ movement associated with the change in anion gradient. It might be argued that the pH buffering within the mitochondria is very low and hence a very small H^+ movement would represent a large change in pH, but this is belied by the pH change observed with nigericin or with uncoupler plus valinomycin (Pressman et al., 1967). Thus, the required H^+ movement associated with anion extrusion implied in the theory of Mitchell (1966) and Chappell and Haarhoff (1967) has not been observed under these particular experimental conditions.

The conclusion of this paper is that K^+ permeability in mitochondria is reversibly variable depending on the metabolic state of the mitochondria. This may have important consequences in determining control phenomena of mitochondrial metabolism. Moreover, this variable permeability raises questions regarding the variability of permeability of other ions such as anions, Ca^{++}, and H^+. The manner through which control is exerted, however, remains to be clarified.

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