Swelling and Contraction of Heart Mitochondria Suspended in Ammonium Chloride*

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ABSTRACT: Isolated beef heart mitochondria do not swell spontaneously in a medium of ammonium chloride at pH 8.3 although the membrane appears to be permeable to both NH_3 and Cl^- under these conditions. In the absence of respiration, however, addition of an uncoupler of oxidative phosphorylation, such as dinitrophenol or *m*-chlorocarbonylcyanidephenylhydrazone, induces a large-amplitude osmotic swelling.

Valinomycin mimics the effect of uncouplers in this regard and the rate of swelling is proportional to the concentration of uncoupler or ionophore added. In the presence of respiration or exogenous adenosine triphosphate uncoupler-dependent ion uptake and swelling are strongly opposed by an energy-dependent contracting reaction and at lower concentrations of uncoupler the swelling can be completely prevented. Passive swelling commences once again when the flow of energy is interrupted by addition of an inhibitor of respiration or of oligomycin in the case of the adenosine triphosphate supported reaction. Elevated rates of respiration and adenosine triphosphatase are observed whether the contraction process occurs or not. These results indicate that both respiration and the utilization of adenosine triphosphate can be coupled to an energy-linked contraction and ion-extrusion process in this medium in the presence of high concentrations of dinitrophenol or *m*-chlorocarbonylcyanidephenylhydrazone. The observations appear to be compatible with the predictions of chemiosmotic coupling models in which uncouplers act as proton conductors through the membrane, but are difficult to reconcile with other suggestions as to the mode of action of uncouplers.

Isolated mitochondria swell rapidly when suspended in an isotonic solution of ammonium acetate in the absence of metabolic energy (Chappell and Crofts, 1966). This osmotic swelling indicates that both the acetate anion and the ammonium cation can penetrate the mitochondrion rapidly in the direction of the chemical gradient. It has been suggested that both of these ions cross the membrane as the un-ionized species and that neutralization of the entering acetic acid by NH_3 results in the rapid accumulation of NH_4^+OAc in the matrix space of the mitochondrion (Chappell and Crofts, 1966; Mitchell and Moyle, 1969b). Since the mitochondrial membrane is relatively impermeable to Cl^- (Chappell and Crofts, 1966; Hunter and Brierley, 1969) and both anion and cation must be permeable to support osmotic swelling, the failure of isolated mitochondria to swell in isotonic NH_4Cl (Chappell and Crofts, 1966; Brierley *et al.*, 1968) at neutral pH is not surprising. Azzi and Azzone (1967a) have noted, however, that the permeability of the mitochondrion to Cl^- increases markedly with increasing pH. Isolated heart mitochondria suspended in NH_4Cl at pH 8.3 do not swell or accumulate ions, however, and since the membrane should be permeable to both NH_4^+ (as NH_3) and to Cl^- under these conditions, it is apparent that additional factors can limit the penetration of these species under these conditions. The present communication presents evidence that pH or membrane potential gradients such as those suggested by Mitchell (1966) may limit the penetration of these ions and that reagents such as valinomycin or uncouplers which alter the permeability of the membrane and therefore alter the status of these gradients can cause large respiration-dependent volume changes in mitochondria suspended in a medium of NH_4Cl. A preliminary report of a portion of this work has been made (Brierley, 1969).

Methods

Beef heart mitochondria were prepared using Nagarse and EGTA as previously described (Settlemire *et al.*, 1968). All experiments were carried out in a medium of 0.1 M NH_4Cl containing 2 mM Tris-chloride, or in some cases 2.5 mM Tris-succinate which served as both buffer and respiratory substrate. The pH was adjusted with small volumes of NH_4OH or HCl. Swelling and contraction were monitored at 25° by absorbance at 546 mμ in a stirred Plexiglass cuvet using an Eppendorf photometer. The oxygen consumption and pH were also recorded simultaneously using a Clark electrode and a combination glass electrode, respectively. For simplicity of presentation, however, these curves are traced in the figures presented only when relevant to the discussion. ATPase activity was estimated by the rate of H^+ production detected by the pH electrode (Nishimura *et al.*, 1962) and by determination of P_i release (Lindberg and Ernster, 1956). Other experimental details are listed with the individual experiments reported.

Results

Isolated heart mitochondria do not swell or accumulate ions when suspended in a lightly buffered medium of 0.1 M

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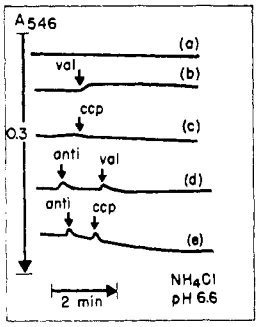

FIGURE 1: Effect of the addition of valinomycin and of CCP to isolated heart mitochondria respiring in a medium of 0.1 M ammonium chloride. Mitochondria (5 mg of protein) were added to 8 ml of a medium of NH_4Cl (100 mM) containing Tris-succinate (2 mM) and rotenone (4 μg/mg) and treated with either valinomycin (2.5×10^{-7} M or 0.3 μg/mg of protein), CCP (6×10^{-7} M), or antimycin (1.2 μg/ml) as indicated. The final pH of the suspension was 6.6. Swelling and contraction were monitored by changes in absorbance at 546 mμ using an Eppendorf photometer.

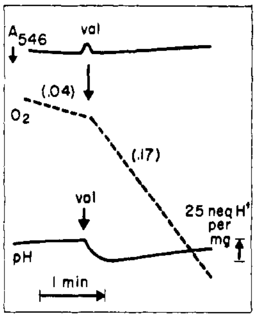

FIGURE 2: Effect of addition of valinomycin on swelling, respiration, and pH changes of heart mitochondria suspended in 0.1 M ammonium chloride. The experiment was carried out under the conditions described for Figure 1 at pH 6.8 with simultaneous measurement of absorbance at 546 mμ, O_2 with a Clark electrode, and pH with a combination glass electrode. The valinomycin concentration was 2.5×10^{-7} M.

NH_4Cl at neutral pH. The study shown in Figure 1 establishes that swelling does not occur under these conditions with respiring mitochondria in the absence of additions (trace a) or in the presence of levels of valinomycin which induce a massive uptake of K^+ and Cl^- from a KCl medium under similar conditions (Brierley, 1969, 1970). Concentrations of CCP[1] which produce maximal stimulation of respiration do not cause swelling in this medium. (trace c). Valinomycin and CCP also produce no swelling in the absence of respiration (traces d and e of Figure 1). It is of interest to note that valinomycin does result in a four- to fivefold activation of respiration when added to mitochondria suspended in 0.1 M NH_4Cl at pH 6.8 (Figure 2). This reagent also produces a pH shift equivalent to the uptake of about 25 mμmoles of H^+/mg of protein when added under these conditions. This response is similar to that obtained when antimycin or CCP are added and is in marked contrast to the cycle of H^+ release and uptake seen when valinomycin is added to respiring heart mitochondria suspended in 0.1 M KCl (Brierley, 1969, 1970).

When the pH of the medium is increased to 8.3 the addition of valinomycin produces striking increases in swelling (Figure 3). With respiring mitochondria valinomycin at 2.5×10^{-7} M produces a swelling which proceeds to only a limited extent and then levels off. At anaerobiosis (or if an inhibitor of respiration is added before anaerobiosis) there is a further rapid swelling (Figure 3A). Swelling induced by higher concentrations of valinomycin is limited to a lesser extent by respiration. If antimycin or cyanide is added before the valinomycin (Figure 3B), rapid swelling ensues immediately with low levels of valinomycin (1×10^{-8} M and below).

A similar situation prevails when CCP or dinitrophenol is added to mitochondria respiring in 0.1 M NH_4Cl at pH 8.3 (Figure 4). Again in this medium, as at neutral pH, there is no swelling in the absence of any addition or when respiration is stopped in the absence of CCP or valinomycin (trace a). Addition of CCP at a suboptimal concentration (based on activation of respiration) results in only a slight swelling as long as respiration continues. If respiration is stopped by anaerobiosis or by addition of an inhibitor, a rapid large-amplitude swelling results (trace b). Under these conditions the influx of ions and osmotic swelling appears to be opposed by a respiration-dependent reaction. This antagonism is more apparent when higher concentrations of CCP are added (Figure 4A, trace c). CCP at 3×10^{-6} M results in a rapid swelling which is followed spontaneously by a respiration-dependent contraction. At anaerobiosis or upon addition of antimycin a further rapid swelling occurs. The contraction phase of this reaction is abolished by antimycin or cyanide, and if one of these inhibitors is present before the addition of the uncoupler, a rapid, large-amplitude swelling results (Figure 4B) with no tendency toward contraction in evidence. The swelling which is obtained under these conditions is a function only of the concentration of uncoupler added.

Mitochondria which have been swollen in the absence of respiration in 0.1 M NH_4Cl at pH 8.3 by the addition of CCP contract rapidly when treated with ATP (Figure 5). This ATP-dependent contraction is abolished if oligomycin is added before the ATP and the mitochondria, once contracted

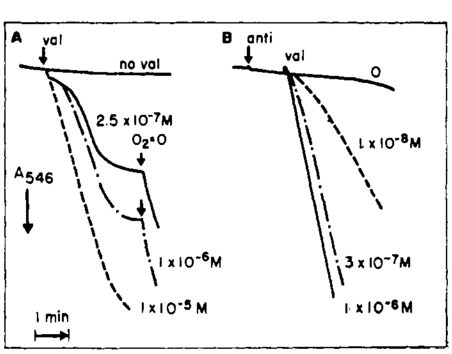

FIGURE 3: Effect of valinomycin addition on swelling of heart mitochondria suspended in 0.1 M ammonium chloride at pH 8.3. The experiment was carried out under the same conditions as described in Figure 1 except that the pH was adjusted to 8.3 by the addition of NH_4OH. The swelling response was recorded in the absence (A) and the presence (B) of antimycin (1.2 μg/ml) and the indicated concentrations of valinomycin.

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[1] The abbreviation used is: CCP, *m*-chlorocarbonylcyanidephenylhydrazone.

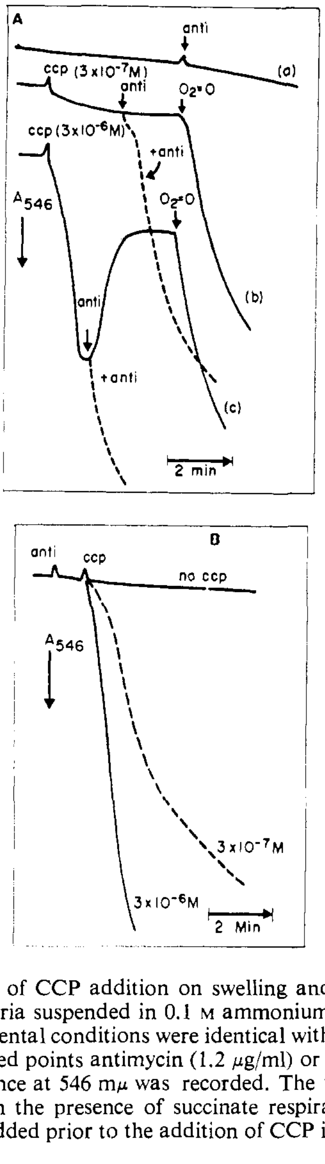

FIGURE 4: Effect of CCP addition on swelling and contraction of heart mitochondria suspended in 0.1 M ammonium chloride at pH 8.3. The experimental conditions were identical with those of Figure 3. At the indicated points antimycin (1.2 μg/ml) or CCP was added and the absorbance at 546 mμ was recorded. The traces in part A were obtained in the presence of succinate respiration; in part B antimycin was added prior to the addition of CCP in order to block respiration.

under these conditions with ATP as the energy source, swell rapidly if treated with oligomycin (Figure 5). The swollen particles contract in a respiration-dependent reaction if a respiratory substrate is added at this point. If respiration ceases due to addition of cyanide, antimycin, or anaerobiosis, the CCP-dependent swelling again predominates (Figure 5).

The extent of the cyclic swelling and contraction reaction induced by the addition of increasing concentrations of CCP to mitochondria respiring with succinate in this medium has been compared with the rate of respiration (Figure 6A). It is apparent that concentrations of uncoupler which induce maximum rates of respiration (3×10^{-6} M) are coupled to a very extensive respiration-dependent contraction reaction. Even at very high concentrations of uncoupler (10^{-4} M) there is a visible tendency for the swollen mitochondria to contract with continued respiration.

When increasing concentrations of CCP are added to mitochondria suspended in 100 mM ammonium chloride at pH 8.3 in the presence of 2.5 mM Tris-ATP (Figure 6B), the uncoupler-dependent ATPase is activated. The rate of ATPase as estimated from the slope of pH traces (not shown) was essentially identical at concentrations of CCP above 1×10^{-6} M. ATPase activity was also estimated from release of P_i and these data (Figure 6B) confirm that nearly maximum ATPase rates are obtained under conditions in which there is considerable oligomycin-sensitive contracting activity opposing the uncoupler-dependent swelling which is seen in the absence of a source of energy under these conditions.

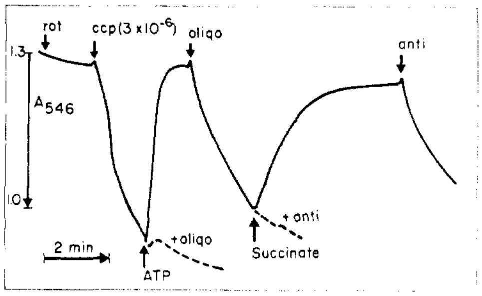

FIGURE 5: Energy requirement for contraction of mitochondria treated with CCP in a medium of 0.1 M ammonium chloride at pH 8.3. Mitochondria (5 mg of protein) were suspended at 25° in a 8 ml of ammonium chloride (100 mM, pH 8.3) containing Tris-chloride (2 mM) and the absorbance at 546 mμ was recorded. Where indicated the following additions were made: rotenone (1 μg/mg), CCP (3×10^{-6}M), Tris-ATP (2.5 mM), oligomycin (2 μg/mg of protein), Tris-succinate (2.5 mM), and antimycin (1 μg/mg). The dashed traces show the effect of the addition of oligomycin before ATP and antimycin before the succinate.

Recent studies indicate that nitrate ions can readily penetrate the membrane of submitochondrial particles at neutral pH (Cockrell, 1969; Montal *et al.*, 1969), and that NH_4NO_3 uncouples phosphorylation in these particles. The study shown in Figure 7 establishes that mitochondria do not swell when suspended in a medium of 100 mM ammonium nitrate at pH 7. Substantial passive swelling is initiated in this medium by the addition of either CCP or valinomycin, however. Addition of ATP establishes an oligomycin-sensitive contracting reaction which opposes the CCP-dependent swelling (Figure 7A, trace d). In a medium of 100 mM KNO_3, rotenone-treated heart mitochondria fail to swell upon the addition of an uncoupler, but will swell passively if treated with valinomycin (Figure 7B). In the presence of respiration mitochondria suspended in KNO_3 fail to swell until treated with valinomycin, and in the presence of the ionophore the cyclic swelling and contraction reaction seen in a KCl medium is obtained (*cf.* Brierley, 1970).

Discussion

The mechanism of action of dinitrophenol and other uncouplers of oxidative phosphorylation remains a subject of some controversy (for recent reviews, see Greville, 1969, Lardy and Ferguson, 1969, and the papers and discussions in Papa *et al.*, 1969). The present work establishes that in a medium of ammonium chloride at pH 8.3, both respiration and exogenous ATP can be coupled to an energy-dependent contraction of the mitochondrion in the presence of "uncoupling" levels of CCP or dinitrophenol. Uncoupler-dependent passive swelling immediately predominates when

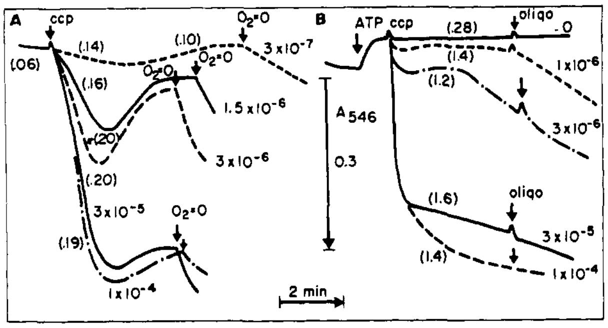

FIGURE 6: Effect of CCP concentration on the swelling cycle in the presence of respiration (A) and exogenous ATP (B). The experimental conditions were identical with those of Figure 5. Rotenone and succinate (2.5 mM) were present in A; rotenone and Tris-ATP (2.5 mM) in B. The final concentration of CCP added is indicated in each swelling trace. In part A the rate of respiration in microatoms of O_2 per minute per milligram of protein is shown in parentheses. The rates were linear to anaerobiosis after the addition of CCP except at the lowest concentration of uncoupler (3×10^{-7} M). In part B the amount of P_i released per milligram of protein in 4 min, as determined by the method of Lindberg and Ernster (1956), is shown in parentheses. All of the ATPase activity was sensitive to oligomycin (2 μg/mg).

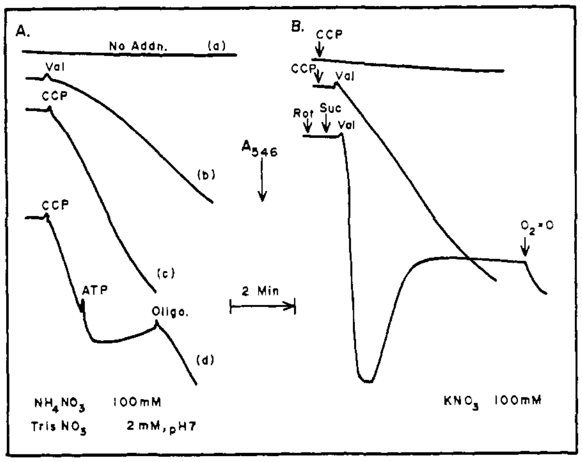

FIGURE 7: Swelling and contraction of rotenone-treated heart mitochondria suspended in (A) a medium of NH_4NO_3 (100 mM) and Tris-nitrate (2 mM, pH 7) and (B) a medium of KNO_3 (100 mM) and Tris-nitrate (2 mM, pH 7). At the indicated points valinomycin (2.5×10^{-7} M), CCP (6×10^{-7} M), Tris-ATP (2.5 mM), and oligomycin (1 μg/mg) were added.

the flow of energy from respiration or ATPase activity is interrupted. Since both respiration and ATPase activity in the presence of the uncoupler proceed at elevated rates typical of uncoupled reactions, we must conclude that the uncoupler is not excluded from its site of activity under these conditions and has actually "uncoupled" or, at least partially uncoupled, the mitochondrial energy transport pathway. Despite the presence of uncoupled respiration and high rates of ATPase, however, the data clearly indicate that both respiration, and ATP-dependent contractile mechanisms are active under the conditions of the present study. These results are difficult to reconcile with suggestions that uncouplers act by hydrolysis of "high-energy" intermediates such as the postulated X∼I (see Chance, 1965, for example). As will be developed below, however, the effects of uncouplers on swelling and shrinking of mitochondria in ammonium chloride fit rather well into the model of uncoupler action proposed by Mitchell (1966) and Mitchell and Moyle (1967) in which the uncoupler acts as a proton conductor in the coupling membrane.

Entrance of NH_3 and Cl^- into the matrix volume of the mitochondrion from a suspending medium of 100 mM ammonium chloride is favored by a large concentration gradient, since neither of these components is present in significant amounts in mitochondria as normally isolated. The rapid osmotic swelling of mitochondria suspended in ammonium acetate indicates that the mitochondrial membrane freely admits NH_4^+, probably as NH_3 (Chappell and Crofts, 1966; Mitchell and Moyle, 1969b). Azzi and Azzone (1967a) have established that mitochondria swell extensively in a medium of KCl at elevated pH provided the membrane is made permeable to K^+ by the addition of valinomycin. These authors have pointed out that accumulation of Cl^- in the matrix in the absence of K^+ permeability is electrogenic and would therefore be limited until valinomycin is added or the membrane is otherwise made permeable to cations. The present study has established that mitochondria suspended in 0.1 M NH_4Cl at pH 8.3 do not swell or accumulate ions either in the presence or absence of respiration until treated with either valinomycin or an uncoupler. Failure of the mitochondrion to swell in the presence of a penetrating anion (Cl^-), a freely permeable potential cation (NH_3), and a large favorable concentration gradient strongly suggests that the penetration of these species is limited by the membrane potential (Mitchell and Moyle, 1969a) or other charge considerations.

A possible explanation for this failure to obtain an accumulation of ions and osmotic swelling under these conditions would be available if it is assumed, in accord with the postulates of the chemiosmotic coupling hypothesis (Mitchell, 1966), that the membrane has only limited permeability to OH^-. Entrance of NH_3 into the matrix would then be followed by the establishment of the equilibrium:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \quad (1)$$

The direction and extent of this reaction would be controlled by the intramitochondrial pH which in turn is reflected in the magnitude of the membrane potential if Mitchell's (1966) model is correct.

The diagrams shown in Figure 8A depict the features of this system at neutral pH. Here swelling is limited in all cases by failure of the Cl^- to penetrate and entrance of NH_3 is limited by the negative charge and alkaline pH of the matrix which favor reversal of eq 1 in the presence of respiration. In the absence of a source of energy the buffering power of the internal components would prevent extensive NH_4^+ and OH^- buildup. Valinomycin appears to act on membrane systems by virtue of its ability to form lipid-soluble ion complexes (Pressman *et al.*, 1967). Although there is some question as to how effectively valinomycin interacts with NH_4^+ (see Henderson *et al.*, 1969) the present results would be explained if the antibiotic permitted the flow of NH_4^+ into the matrix and neutralized the metabolically generated OH^-. A backflow of NH_3 would then establish the same cycle which has been postulated for uncouplers (Mitchell, 1966) and result in

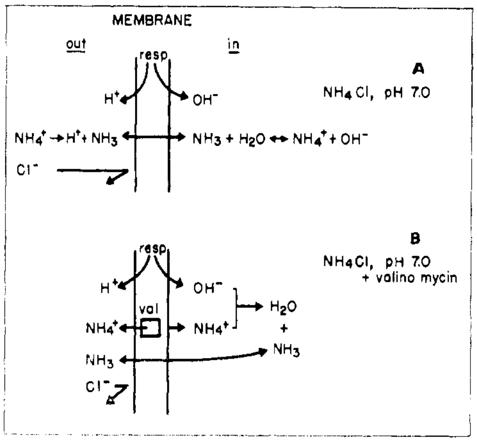

FIGURE 8: Postulated model for the penetration of NH_3 and NH_4^+ into mitochondria at pH 7 in the presence of respiration.

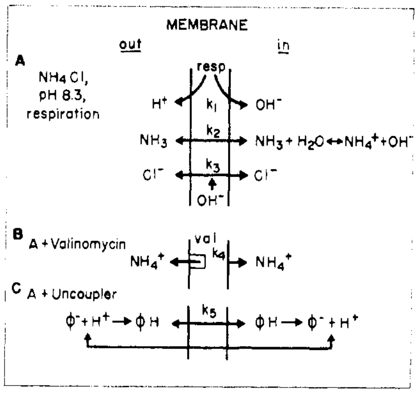

FIGURE 10: Postulated reactions which can account for biphasic swelling and contraction of respiring mitochondria in $NH_4^+Cl^-$ at pH 8.3. See the text for a more complete description.

increased respiration (Figure 2) by equilibrating H^+ across the membrane (Figure 8B). It should be noted that addition of valinomycin under the present conditions produces a pH shift typical of uncouplers and does not result in the cycle of H^+ release which has been related to increased permeability to Cl^- in the previous study (Brierley, 1969, 1970).

Support for this model is available in recent reports that valinomycin markedly uncouples submitochondrial particles in the presence of NH_4^+ salts (Cockrell and Racker, 1969; Montal *et al.*, 1969). These authors have suggested that NH_3 enters these particles and reacts with the H^+ produced by respiration (in the interior of the particle, since the membranes appear to be oriented in the opposite direction to that of intact mitochondria). Addition of valinomycin would then provide a channel for NH_4^+ out of the interior of the particle and result in uncoupling. These studies employed release of dicyclohexylcarbodimide-controlled respiration and ATP-P_i exchange activity (Cockrell and Racker, 1969; Montal *et al.*, 1969) rather than osmotic swelling responses as in the present study, and are clearly in line with the model presented here.

At alkaline pH the entrance of Cl^- is no longer the limiting feature of the system, but as shown in Figure 9A the accumulation of $NH_4^+Cl^-$ in the interior is electrogenic and limited by the alkaline pH and internal buffering both in the presence and the absence of respiration. One should bear in mind that this would not be a restricting feature if the membrane were freely permeable to OH^-. In the absence of respiration or ATP (assumed by the chemiosmotic coupling hypothesis to be able to generate a similar H^+ gradient and membrane potential to that of respiration) the addition of uncoupler (Figure 9B) or valinomycin (Figure 9C) establishes permeability to either NH_4^+ or H^+ in the case of the former and to NH_4^+ in the case of the ionophore and both cases lead to the accumulation of $NH_4^+Cl^-$ without the restriction imposed by the OH^- component. The effect of the uncoupler would be identical if it functioned as a H^+ carrier or as a cation carrier, but since high concentrations of CCP induce only slow swelling in a medium of 0.1 N NaCl at pH 8.3 and no swelling in 0.1 M KCl at this pH (G. P. Brierley, unpublished) the role of H^+ carrier seems more likely.

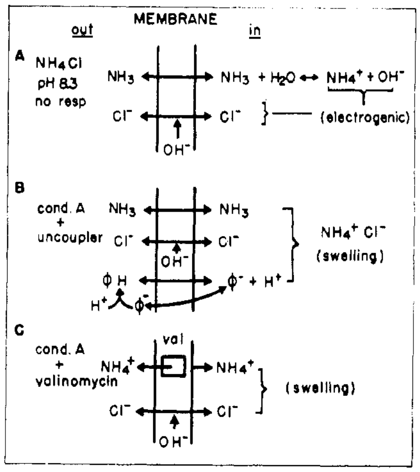

FIGURE 9: Postulated model for $NH_4^+Cl^-$ swelling of mitochondria at pH 8.3 in the absence of respiration.

In the case of addition of valinomyicn or uncoupler to respiring mitochondria suspended in NH_4Cl at pH 8.3 the situation shown in Figure 10 might be supposed to prevail. Here the relative rates of the following reactions will determine whether swelling or contraction occurs: (a) the rate of respiration or ATP-dependent H^+ and OH^- separation across the coupling membrane, (b) the rate of entrance and exit of NH_3 which will be controlled by the interior pH, (c) the rate of entrance and exit of Cl^- under the influence of elevated pH, and (d) the rate of entrance of either NH_4^+ or H^+ under the influence of uncoupler or valinomycin. Clearly, in the presence of high enough concentrations of uncoupler the rate of proton entrance (k_5 in Figure 9) will supply H^+ at a rate which can both neutralize the metabolic OH^- (k_1) and the OH^- from NH_3 entrance (k_2) and osmotic swelling due to $NH_4^+ + Cl^-$ accumulation will be the predominant result. At lower levels of uncoupler k_5 would be of insufficient magnitude to neutralize both sources of OH^- (k_1 and k_2) and no swelling would occur. This situation would result in increased rates of respiration and ATPase, however, since the negative charge and pH of the interior would both be lowered by the influx of protons.

At intermediate levels of uncoupler the relative rates of these processes could easily account for observations such as those of Figures 4 and 6 where first swelling and then contraction predominate. It should be noted that to support contraction the rate of the metabolic component (k_1) must exceed the rate of proton or cation entrance (k_4 or k_5). It is also possible that an exchange diffusion system which equilibrates external H^+ with internal cation (*cf.* Mitchell, 1966, and the discussion in the previous paper, Brierley, 1970) could contribute to the observed contraction.

Many of the considerations developed here also have been shown to apply to the uptake of NH_4^+ by chloroplasts if the opposite orientation of the metabolic H^+ production is taken into account (Crofts, 1967, 1968). Chappell and Crofts (1965) have noted an uncoupler-like response following the addition of NH_4^+ to mitochondria swollen with K^+ and P_i in the presence of gramicidin. These authors interpreted these results in terms of the ability of NH_4^+ to acidify the interior of the mitochondrion in the presence of gramicidin.

Studies of swelling of mitochondria in ammonium nitrate (Figure 7) also support the model developed for swelling in Cl^- media. In this case it appears that the mitochondrial membrane is penetrated by NO_3^- at neutral pH in the presence of a permeant cation (such as K^+ in the presence of valinomycin) and in the absence of restrictive effects of the membrane potential or of the internal concentration of OH^- The response in this medium is essentially the same as that in NH_4Cl at pH 8.3 as developed above and in the model in Figure 10. The experiments with nitrate establish that ATP-dependent contraction can be obtained in the presence of high concentrations of CCP at neutral pH as well as at pH 8.3 and support the contention that the elevated pH is necessary only to promote the permeability of Cl^-.

A number of studies of respiration-dependent contraction of swollen mitochondria have noted a perplexing discrepancy between the concentration of uncoupler required to inhibit contraction and the effective level of these reagents in abolishing other energy-linked reactions (Crofts and Chappell, 1965; Blair and Sollars, 1967; Azzi and Azzone, 1967a,b; Hansen *et al.*, 1968; Scott *et al.*, 1970). A portion of the uncoupler-dependent increment in respiration seems to be capable of supporting contraction in at least one of these cases (Hansen *et al.*, 1968). This result is compatible with the models developed in this and the previous study (Brierley, 1970) since it is assumed that contraction in media other than NH_4^+ salts depends on the influx of H^+ in exchange for internal K^+ *via* the K^+/H^+ antiport (Mitchell, 1966) which can be considered as a type of controlled uncoupling. One could speculate that under certain conditions the antiport channel for H^+ influx could be effective in the presence of suboptimal amounts of an extraneous proton conductor.

Mitchell and Moyle (1967) have established in oxygen-pulse experiments that uncouplers do not affect the initial translocation of protons, but that these reagents do accelerate the rate of reequilibration of protons. These results like those of the present study do not appear to be compatible with the postulated interaction of uncouplers with a chemical intermediate of oxidative phosphorylation (*cf.* discussion in Greville, 1969).

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