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Energy-Linked Alteration of the Permeability of Heart Mitochondria to Chloride and Other Anions*

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ABSTRACT: Isolated beef heart mitochondria suspended in 0.1 M KCl do not swell when treated with valinomycin in the absence of a source of energy at neutral pH. Extensive osmotic swelling and uptake of K^+ and Cl^- can be induced under these conditions by either (a) increasing the pH to above 8 or (b) the addition of either a respiratory substrate or exogenous adenosine triphosphate at neutral pH. The swelling in a medium of neutral pH is preceded by a cycle of H^+ extrusion into the medium followed rapidly by an uptake of H^+ so that the initial pH of the reaction medium is reestablished. The K^+- and Cl^--loaded swollen mitochondria contract spontaneously and extrude ions in an energy-linked reaction in which no visible pH changes occur. In the presence of a "permeant" anion such as acetate or phosphate, energy-linked swelling rather than contraction is observed under these conditions. These data suggest that energy-linked pH changes in either the membrane itself or in the matrix compartment can alter the permeability of the membrane to Cl^- and other nonpermeant anions in much the same way as an externally imposed high pH. These observations appear to be compatible with the predictions of Mitchell's chemiosmotic coupling hypothesis.

Isolated heart mitochondria appear to be impermeable to Cl^- at neutral pH (Chappell and Crofts, 1966; Brierley *et al.*, 1968), but admit large amounts of this anion as the pH is increased (Azzi and Azzone, 1967a). If the membrane is made permeable to K^+ by the addition of valinomycin under conditions of Cl^- permeability, a massive uptake of ions and osmotic swelling results (Azzi and Azzone, 1967a). We have recently reported that an extensive osmotic swelling which is characterized by the uptake of both K^+ and Cl^- and by expansion of the matrix as seen in electron micrographs can be obtained at neutral pH when respiring mitochondria are treated with valinomycin or gramicidin (Hunter *et al.*, 1969). In a recent preliminary report (Brierley, 1969) it was suggested that this permeability to Cl^- at neutral pH was the result of a transient membrane alkalization which resulted from a valinomycin- and K^+-dependent proton gradient developed by respiring mitochondria. The present communication presents the details of these experiments and a more extensive development of the thesis that a respiration-dependent separation of H^+ and OH^- across the mitochondrial membrane as proposed by Mitchell (1966; Mitchell and Moyle, 1969a) can account for the observed osmotic swelling and for the energy-linked extrusion of ions and contraction which ensues spontaneously in the presence of continued respiration.

Methods

Beef heart mitochondria were prepared by Nagarse treatment in the presence of EGTA[1] (Hatefi *et al.*, 1961; Settlemire *et al.*, 1968) and contained less than 5 mμmoles of Ca^{2+}/mg of protein. The basic incubation medium consisted of KCl (100 mM) and Tris-succinate (2 mM) without other additions. Variations in the composition of this medium are noted with the individual experiments reported. Mitochondria (5 mg of protein in 0.2 ml of 0.25 M sucrose) were added to 8 ml of the incubation medium which was stirred at 25° in a Plexiglass cuvet. Swelling and contraction, pH, and O_2 uptake were

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[1] The abbreviations used are: CCP, *m*-chlorocarbonylcyanidephenylhydrazone; EGTA, ethyleneglycolbis(aminoethyl)tetraacetic acid; val, valinomycin.

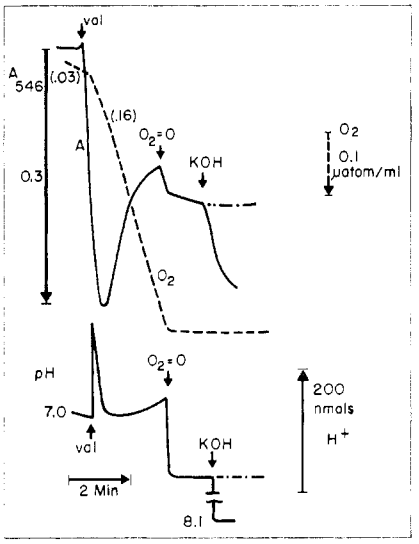

FIGURE 1: Valinomycin-dependent changes in swelling, pH, and respiration of isolated heart mitochondria suspended in 0.1 M KCl. Beef heart mitochondria (5 mg of protein) were treated with rotenone and added to 8 ml of a medium of KCl (0.10 M) and Tris-succinate (2 mM, pH 7.0). The final concentration of sucrose (added with the mitochondria) was 6 mM. The reaction was carried out at 25° in a stirred Plexiglass cuvet mounted on an Eppendorf photometer and equipped with a Clark oxygen electrode and a combination glass electrode. Absorbance at 546 mμ was recorded as a measure of swelling and contraction. At the indicated points valinomycin (2.5×10^{-7} M) or sufficient 0.1 M KOH to increase the pH to 8.1 were added. The dashed traces show the rate of oxygen uptake in microatoms per minute per milligram of protein.

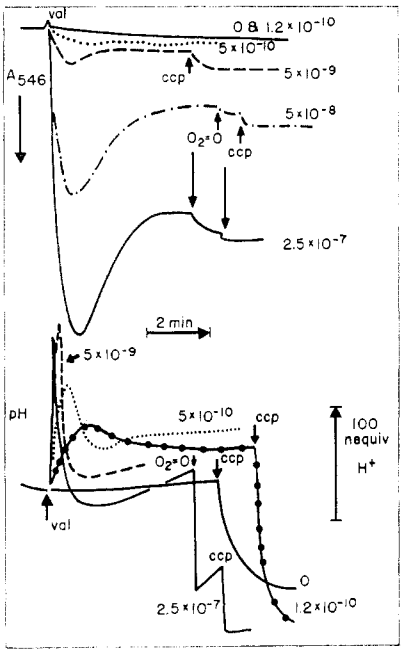

FIGURE 2: The effect of increasing concentrations of valinomycin on swelling, contraction, and pH changes of heart mitochondria suspended in 0.1 M KCl. Experimental conditions were identical with those of Figure 1 with the indicated concentrations of valinomycin. CCP was added to 6×10^{-7} M where indicated.

monitored simultaneously by absorption at 546 mμ using the Eppendorf photometer, a Thomas 4858 combination electrode, and a Clark electrode, respectively.

For experiments in which the K^+, Cl^-, and water content was monitored, the mitochondria were rapidly centrifuged and extracted with $HClO_4$ as described by Hunter and Brierley (1969). K^+ content was estimated by atomic absorption spectroscopy, Cl^- by the content of $^{36}Cl^-$, and water by tritiated water distribution.

Results

Induction of a Cycle of Swelling and Contraction of Heart Mitochondria Suspended in 0.1 M KCl. Isolated heart mitochondria do not swell or accumulate ions when they respire in a lightly buffered medium of 0.1 M KCl. In the absence of respiration the addition of valinomycin or gramicidin to mitochondria suspended in this medium also does not induce ion uptake or osmotic swelling at pH 7.0. However, addition of valinomycin (2.5×10^{-7} M) to a suspension of respiring mitochondria under these conditions (Figure 1) results in (a) a rapid cycle of acidification of the medium followed by a spontaneous alkalinization so that the pH at the end of the cycle is very close to the initial value, (b) a fivefold activation of respiration, and (c) a rapid, large amplitude swelling. This phase of the reaction is complete in about 10–15 sec at 25° and in the period which follows there is little change in pH, respiration remains at the elevated rate, and the particles now contract in a respiration-dependent reaction (Figure 1). At anaerobiosis the medium becomes more alkaline to the extent of about 25–30 mμmoles of H^+ taken up (or base released) and there is a rapid, but brief, swelling followed by a long period in which little further volume adjustment takes place. Addition of KOH to bring the pH to 8.1 at this point results in further large-amplitude swelling (Figure 1).

The swelling obtained under these conditions resembles that reported by Azzi and Azzone (1967a) for valinomycin-induced KCl uptake and swelling at elevated pH in the absence of a source of energy. In the present study, as in the reports of Azzi and Azzone (1967a,b), analysis of total water, K^+, and $^{36}Cl^-$ contents of isolated pellets of swollen mitochondria indicates an osmotic swelling in response to salt uptake. The total water content of centrifuged pellets of mitochondria increased by 1.5 μl/mg of protein, K^+-permeable water by 1.8 μl/mg, and Cl^--permeable water by 2.5 μl/mg. The fact that the Cl^- content of these pellets increases to a greater extent than does the K^+ indicates that the normal permeability barrier of the mitochondrion to Cl^- at pH 7.0 is no longer present and that a redistribution of anions has probably occurred. Electron micrographs of mitochondria swollen under these conditions (Hunter *et al.*, 1969, Figures 13 and 14) show that the matrix of the mitochondrion expands as a result of this uptake of K^+ and Cl^- in the same way as it does following accumulation of K^+ and permeant anions such as acetate. Micrographs (G. R. Hunter, unpublished) have also established that the matrix of the bulk of the swollen particles contracts during the energy-linked extrusion of ions and contraction phase of the reaction shown in Figure 1. In agreement with Azzi and Azzone (1967b) our studies show that K^+ and Cl^- are extruded during the respiration-dependent

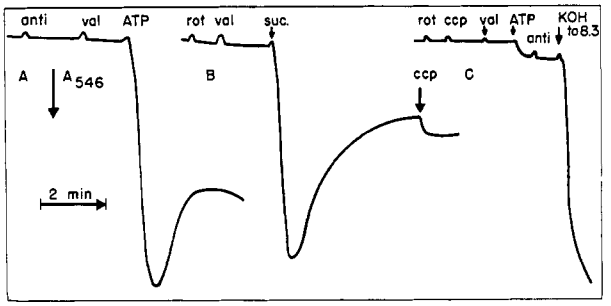

FIGURE 3: Requirements for the swelling phase of the cyclic reaction shown in Figure 1. The reactions were carried out under the conditions described for Figure 1 except that in part B the Tris-succinate was replaced with Tris-Cl (2 mM, pH 7.0) initially and K^+-succinate was added to a final concentration of 2 mM at the point indicated. The concentrations of other additions were as follows: Tris-ATP (2.5 mM), antimycin (2 μg/mg of protein), valinomycin (2.5×10^{-7} M), CCP (6×10^{-7} M), and rotenone (4 μg/mg).

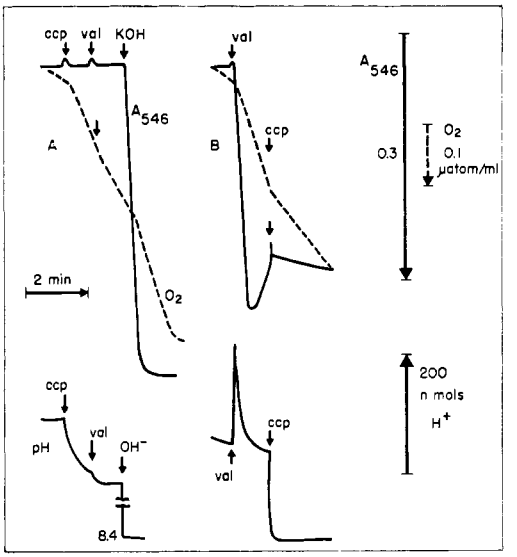

FIGURE 4: Effect of uncouplers on the swelling and contraction phases of the cyclic reaction shown in Figure 1. Additions of CCP (6×10^{-7} M), valinomycin (2.5×10^{-7} M), and sufficient KOH to bring the pH to 8.4 were made where indicated.

contraction of swollen mitochondria. There appears to be little doubt therefore, that the matrix of the mitochondrion accumulates large amounts of Cl^- from a medium of neutral pH during the osmotic swelling phase of this reaction and then spontaneously contracts and expels a portion of the accumulated ions with continued respiration.

Conditions Necessary for Initiating the Swelling Phase of the Reaction. A. VALINOMYCIN CONCENTRATION. The effect of the concentration of valinomycin added under the conditions just described is shown in Figure 2. Optimal swelling is induced by 2.5×10^{-7} M valinomycin (0.3 μg/mg of protein), but it is apparent that major shifts in pH are elicited by concentrations of valinomycin as low as 1×10^{-10} M under these conditions. The magnitude of the valinomycin-induced pH cycle is of course a function of the mixing and response time of the measuring system, and for this reason estimates of the magnitude of the H^+ shifts following the addition of valinomycin under these conditions are rather crude. The cycle seen on traces such as those of Figure 2 amounts to about 30 mμmoles/mg of H^+ extruded (or base taken up) and the magnitude of the cycle observed in the presence of 5×10^{-9} M valinomycin is similar to that seen with a level of valinomycin which induces optimal swelling (2.5×10^{-7} M). The lower level of valinomycin induces little swelling under these conditions, however (Figure 2).

B. SOURCE OF ENERGY. Initiation of the swelling phase of the cyclic reaction requires a source of energy. In the absence of respiration this requirement can be met by exogenous ATP (Figure 3A). The swelling phase, but not the subsequent contraction, can be supported by endogenous respiration in the absence of added substrates. If endogenous respiration is abolished by rotenone, addition of valinomycin fails to cause swelling (Figure 3B). Addition of succinate or ascorbate plus *N,N,N',N'*-tetramethylphenylenediamine under these conditions, however, initiates a cycle of swelling and contraction which is identical with that obtained under the conditions of Figure 1. In the presence of an uncoupler such as CCP (Figure 3C) valinomycin addition does not result in swelling with either respiration or exogenous ATP. A rapid swelling does result under these conditions, however, if the pH is increased to 8.3 by the addition of KOH (*cf.* Azzi and Azzone, 1967a).

Addition of antimycin or an uncoupler prior to valinomycin results in an alkaline shift of about the same magnitude as the pH change seen in the reaction of Figure 1 at anaerobiosis (Figure 4A). Addition of valinomycin at a point after the discharge of this gradient does not produce an acid shift or result in osmotic swelling. In addition, the elevated rate of respiration induced by the addition of CCP is inhibited by the addition of valinomycin (Figure 4A).

The energy requirement for the contraction of mitochondria swollen as the result of KCl uptake has been established by Azzi and Azzone (1967b) and confirmed in the present study. Addition of either an uncoupler or an inhibitor of respiration after the completion of the valinomycin-induced pH cycle (Figure 4B) causes (a) an immediate alkaline shift of slightly larger magnitude than that shown at the anaerobic point in Figure 1, (b) inhibition of the high rate of respiration characteristic of the contraction phase of the reaction, and (c) an immediate cessation of the contraction of the mitochondria. At neutral pH there is little tendency for further swelling at this point by the uncoupled mitochondria (Figure 4B). As expected, however, at elevated pH the particles start to swell when the energy source is removed (Azzi and Azzone, 1967a).

The swelling phase of the reaction is inhibited by the presence of nonpermeant solutes such as sucrose and NaCl (Figure 5). A low amplitude cycle of swelling and contraction can be detected even when opposed by the osmotic pressure of 100 mM sucrose, however (Figure 5).

C. pH. When the reaction shown in Figure 1 is carried out at pH 6.0 and 8.3, the results are very similar to those shown for pH 7.0. In both cases a slightly greater swelling is seen and the contraction phase which follows is not as prominent as at neutral pH (Figure 6). At pH 8.3 there is further swelling following anaerobiosis.

The valinomycin- and respiration-dependent acid shift detected by the glass electrode is also very similar at pH 6.0,

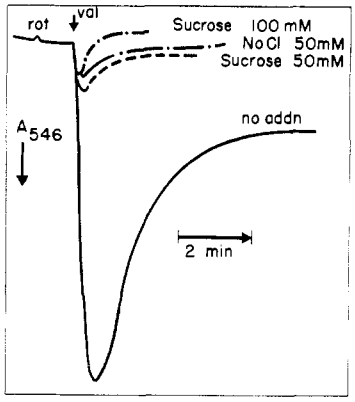

FIGURE 5: Inhibition of swelling by sucrose and by NaCl. The experiment was carried out as described for Figure 1 with the indicated additions of sucrose and of NaCl.

7.0, and 8.3 at 25° (Table I). The magnitude of the alkaline shift at anaerobiosis or on the addition of CCP varies considerably with pH, however, with changes in excess of 50 mμmoles/mg of protein at the lower pH and almost negligible shifts at pH 8.3 (Table I). This result may indicate increased loss of K^+ from the matrix in exchange for H^+ under these conditions (*cf.* Carafoli *et al.*, 1969). In all cases the pH shift appears to be larger than that which would be expected from the release of the low levels of Ca^{2+} present in these preparations.

The valinomycin- and respiration-dependent pH changes and the subsequent osmotic swelling can be slowed down by running the experiment at 11°. Under these conditions sufficient time is available to get well-mixed additions and show proper response time for the pH measuring system. A series of such experiments is shown in Figure 7. Respiration-dependent acid shifts are observed on addition of valinomycin and alkaline shifts occur when CCP or antimycin are added.

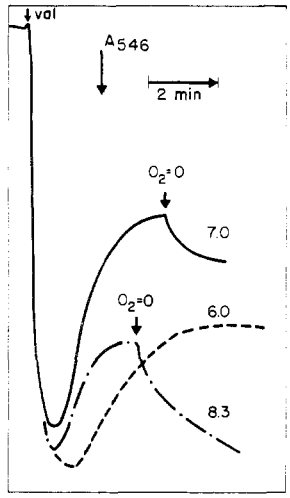

FIGURE 6: Effects of initial pH on the cyclic reaction shown in Figure 1. The reaction was carried out at 25° under the conditions described for Figure 1. The indicated pH was established by the addition of small volumes of 1 M HCl or KOH to the medium used in Figure 1.

TABLE I: pH Shifts Induced by Valinomycin in Various Media.[a]

Suspending Medium, KCl (mM)	Initial pH	Temp (°C)	Valinomycin-Dependent H^+ Rel (mμmoles/mg)	H^+ Uptakes on Addn of CCP (mμmoles/mg)
100	6.0	25	27	50
100	7.0	25	35	28
100	8.3	25	20	−4
100	6.0	10	31	59
100	7.0	10	40	37
100	8.3	10	22	11
300	7.0	10	40	18
100	7.0	10	40	37
25	7.0	10	22	32

[a] The reactions were carried out at the temperature shown in the indicated media containing Tris-succinate (2 mM) and rotenone as described in the legend for Figure 1. The magnitude of the acid shift induced by valinomycin (2.5×10^{-7} M) is tabulated along with the magnitude of the alkaline shift on addition of CCP (6×10^{-7} M) after the completion of the pH cycle. Each experiment was quantitated by titration with standard HCl after the CCP-dependent alkaline shift.

The magnitude of these pH changes is very close to those seen at 25° (Table I) for pH 6.0, 7.0, and 8.3. It should be noted that there is insufficient energy available from succinate respiration at this temperature to support the energy-linked contraction phase of the reaction.

The relationship between the pH cycle and the induction of osmotic swelling is clarified by the slower reaction at 11° and by the absence of the contraction phase. Addition of CCP just after initiating the pH cycle by the addition of valinomycin (Figure 7B) results in an immediate alkaline shift to about the same end point as is seen after a complete cycle followed by addition of antimycin (compare Figure 7B with 7A) and prevents the osmotic swelling. If CCP is added at the point of maximum deflection in the valinomycin-induced acid cycle (Figure 7C) a greater alkaline shift results, but about the same end point is attained as in the two previous experiments. In this case swelling can be seen to commence during the acid shift which follows the addition of valinomycin and, when the CCP is added, considerable further swelling occurs (Figure 7C). When CCP is added during the phase of the pH cycle in which the acid produced is being reequilibrated, the alkaline shift again reaches the same final point. In this case (Figure 7D) the swelling after the additions of CCP proceeds to about the same extent as in the absence of the uncoupler (Figure 7A).

Swelling and Contraction Cycles in Hypertonic and Hypotonic KCl Solutions. High concentrations of KCl in the suspending medium (300 mM) result in much less-pronounced swelling than is seen in 100 mM KCl following the addition of valinomycin, and no tendency to contract can be detected in this medium (Figure 8A). The shifts in pH and the activation

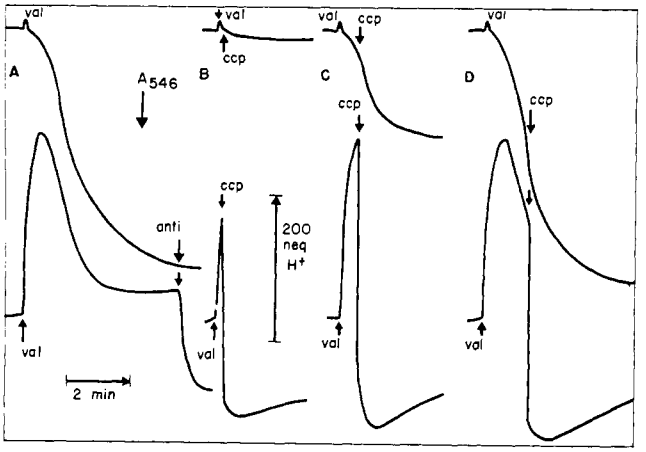

FIGURE 7: Valinomycin-induced swelling and pH changes at 11° in 100 mM KCl. The reactions were carried out as described in Figure 1 except at 11°. CCP (6×10^{-7} M) was added at various points in the reaction sequence as indicated.

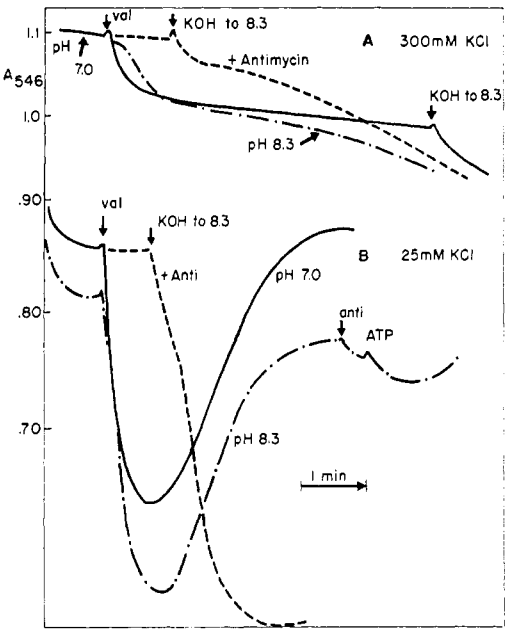

FIGURE 8: Valinomycin-induced swelling in hypertonic and hypotonic KCl. The traces shown in part A were obtained in a medium of 300 mM KCl containing 2 mM Tris-succinate. The experiments were carried out as described for Figure 1. Where indicated KOH was added to bring the pH to 8.3. Part B was carried out in 25 mM KCl containing 2 mM Tris-succinate. The valinomycin concentration was 2.5×10^{-7} M; antimycin 2 μg/mg of protein.

of respiration following the addition of valinomycin are the same as those shown in Figure 1 for 100 mM KCl at both 25 and 10° (Table I). The swelling which results from increasing the pH to 8.3 with KOH in 300 mM KCl is also much less than that obtained in 100 mM KCl (Figure 8A).

Mitochondria suspended in hypotonic solutions of KCl (25 mM) swell in response to the decreased tonicity without further addition. These hypotonically swollen mitochondria respond to the addition of valinomycin in exactly the same way as do isotonic suspensions. The magnitude of the pH shift in 25 mM KCl is somewhat less than that obtained in 100 mM KCl (Table I) and the respiration rates both before and after the addition of valinomycin are lower in the lower tonicity medium. Addition of valinomycin at either pH 7.0 or 8.3 results in a cycle of further extensive swelling followed by energy-linked contraction in 25 mM KCl (Figure 8B). The contraction in this medium is of the same or slightly greater magnitude than the swelling phase, however. Rapid, passive swelling is obtained at pH 8.3 in this medium with valinomycin-treated mitochondria (Figure 8B).

Effects of Altered Tonicity on the Valinomycin-Induced Cycle of Swelling and Contraction. Mitochondria treated with excess valinomycin at pH 7.0 should be permeable to K^+ but not to Cl^-. The effect of the addition of 75 mM KCl to mitochondria at various stages in the valinomycin- and respiration-dependent cycle of swelling and contraction in 25 mM KCl was therefore investigated in an effort to test the permeability of the mitochondrion to Cl^- at each stage. If respiration is inhibited by the addition of antimycin or if CCP is added at the point of maximum swelling (Figure 9A), the mitochondria remain swollen and no volume change in either direction can be detected. Addition of a pulse of KCl to mitochondria in this condition (75 mM added so that the final concentration of KCl in the suspending medium is 100 mM) results in a rapid contraction of very nearly the same magnitude as that obtained with the addition of a like amount of NaCl. This response indicates that under these conditions the particles are not permeable to Cl^-. If the pH is increased to 8.3 so that Cl^- permeability becomes appreciable, then a rapid swelling results (Figure 9A). When a pulse of 75 mM KCl is added to respiring mitochondria which have been swollen in 25 mM KCl by the addition of valinomycin, the osmotic contraction which results is much less than in the absence of respiration and is immediately followed by a short period of further swelling and then by respiration-dependent contraction (Figure 9B). If the pulse of KCl is added during the valinomycin-induced swelling phase in this medium, the osmotic contraction can be seen to begin, but is reversed almost immediately so that the net effect is a major increment

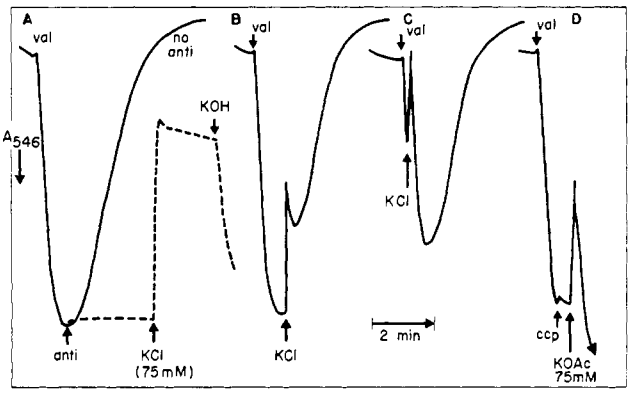

FIGURE 9: Osmotic response of heart mitochondria swollen in 25 mM KCl by the valinomycin- and respiration-dependent reaction. The experiment was carried out as described for Figure 8B. At the indicated points enough 3 M KCl or KOAc was added to bring the final concentration to 100 mM. See the text for a complete description.

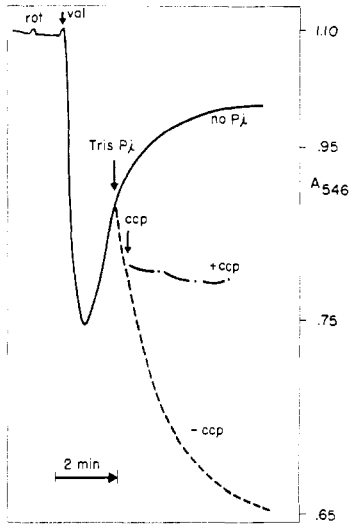

FIGURE 10: Effect of addition of a "permeant anion" on the energy-linked contraction phase of the cyclic reaction shown in Figure 1. The experimental conditions were identical with those of Figure 1 with Tris-phosphate (3 mM) and CCP (6×10^{-7} M) added at the indicated points.

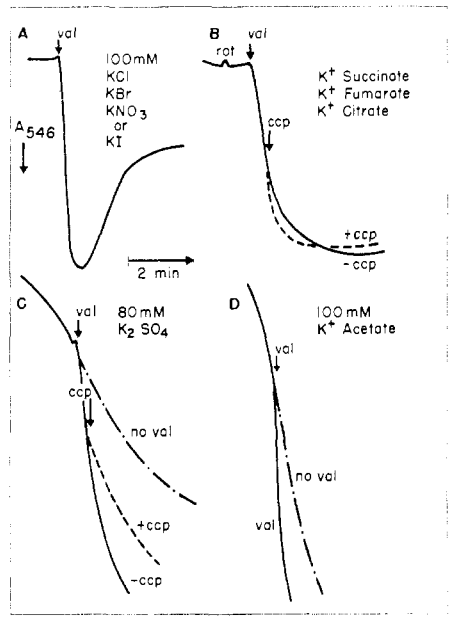

FIGURE 11: Effect of substituting various anions for Cl^- on the valinomycin-induced cycle of swelling and contraction. (A) The absorbance trace obtained upon addition of valinomycin (2.5×10^{-7} M) to a medium of either KCl, KBr, KNO_3, or KI (each 100 mM) containing 2 mM Tris-succinate. All four media give essentially the same trace typified by considerable energy-linked contraction when measured under the conditions of Figure 1. (B) Trace obtained upon addition of valinomycin to a medium of K^+-succinate, K^+-fumarate, or K^+-citrate (each 80 mM) containing 2 mM Tris-succinate and rotenone. (C) Trace obtained in 80 mM K_2SO_4 and 2 mM Tris-succinate. (D) Trace in 100 mM K^+-acetate and 2 mM Tris-succinate. The concentration of CCP added at the indicated points was 6×10^{-7} M. Other experimental conditions were identical with those of Figure 1.

in swelling after the addition of the KCl. The usual respiration-dependent contraction then takes place (Figure 9C). The osmotic response to KCl in the last two cases (Figure 9B,C) resembles the response to a pulse of 75 mM K^+ salt of a permeant anion such as acetate (Figure 9D). Pulses of K^+-acetate result in a rapid osmotic contraction followed by further swelling in the presence of an uncoupler. In the absence of an uncoupler passive swelling in a medium of K^+-acetate by valinomycin- or gramicidin-treated mitochondria occurs much more slowly (see Brierley *et al.*, 1968; Henderson *et al.*, 1969; Mitchell and Moyle, 1969b). In the case of addition of permeant anions, however, no contraction phase occurs regardless of the respiration state. These results are compatible with the suggestion that the mitochondria are permeable to Cl^- during the respiration- and valinomycin-dependent cycle of swelling and contraction, but that in the absence of respiration they are not permeable to Cl^-.

Effect of Substitution of Other Anions for Cl^-. As just noted in the previous section, addition of a permeant anion such as acetate or phosphate to respiring mitochondria which have been treated with valinomycin results in swelling which is not followed by energy-linked contraction. The study shown in Figure 10 shows that addition of 3 mM Tris-phosphate to a suspension of mitochondria in 100 mM KCl which are undergoing energy-linked contraction results in an immediate energy-linked swelling. The swelling which results under these conditions requires respiration and is inhibited by the addition of uncouplers (Figure 10). It is apparent, therefore, that the ability of the mitochondria to swell or contract under these conditions depends strongly on the anionic composition of the suspending medium. Addition of ADP in addition to phosphate under these conditions does not inhibit the swelling. ADP is not phosphorylated to ATP under these circumstances or under any of the conditions tested after treatment with 2.5×10^{-7} M valinomycin, however.

The cycle of energy-initiated swelling followed by energy-linked contraction typical of the KCl medium of Figure 1 is also obtained when a 100 mM solution of the K^+ salt of Br^-, I^-, or NO_3^- is used as the suspending medium (Figure 11A). Nearly identical swelling, pH, and respiration traces are obtained in all of these media (KCl, KBr, KI, and KNO_3). If the Cl^- in the suspending medium is replaced by succinate, fumarate, or citrate (Figure 11B) the respiring mitochondria do not swell until valinomycin is added. In each case succinate respiration was the source of energy and rotenone was present to minimize further conversion of the substrate anions. Following the addition of valinomycin each of these anions supports extensive osmotic swelling which is not affected by the addition of CCP (*cf.* Figure 7 for effects of CCP on the Cl^- system), but in each case there is little tendency toward contraction on further incubation (Figure 11B). When the Cl^- of the suspending medium is replaced with a permeant anion such as sulfate (Figure 11C) or acetate (Figure 11D), the spontaneous, respiration-dependent swelling which occurs (*cf.* Brierley *et al.*, 1968) is enhanced by the addition of valinomycin. CCP inhibits swelling under these conditions in the sulfate medium but, as has been previously documented, the combination of CCP with valinomycin enhances passive swelling of mitochondria in the presence of a permeant anion (see Brierley *et al.*, 1968; Henderson *et al.*, 1969).

Effect of Substituting Na^+ for K^+. Gramicidin, like valino-

mycin, induces the cycle of swelling and contraction shown in Figure 1 in a medium of 100 mM KCl (Figure 12). Since gramicidin is an ionophore with much less specificity for K^+ than valinomycin (Pressman, 1965) the effect of replacing the K^+ of the suspending medium with Na^+ was tested using this antibiotic. In a medium of 100 mM NaCl gramicidin elicits a somewhat reduced cycle of swelling and contraction as compared with the KCl medium (Figure 12C). As expected, valinomycin has no effect in this medium (Figure 12D). In a mixture of 50 mM NaCl and 50 mM KCl valinomycin induces a very slight swelling and contraction cycle (Figure 12E), whereas gramicidin induces a swelling which is less than that seen in either 100 mM KCl or 100 mM NaCl. In agreement with the recent report of Wenner and Hackney (1969) we have noted enhanced swelling in a medium of NaCl if both ATP and substrate are present. Heart mitochondria swell extensively in the absence of a source of energy when suspended in isotonic Na^+-acetate (Brierley *et al.*, 1968), a result which suggests that Na^+ can readily traverse the mitochondrial membrane. It should be noted, however, that passive swelling does not occur in the absence of gramicidin when mitochondria respire in 0.1 M NaCl at pH 8.3, a condition in which Cl^- is permeable. The permeability of the membrane to Na^+ therefore appears to be complex and strongly dependent on the anionic composition of the medium. This problem will be discussed more extensively elsewhere.

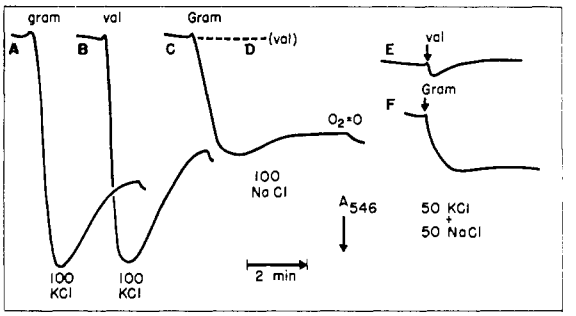

FIGURE 12: Gramicidin-induced swelling of heart mitochondria suspended in K^+- and Na^+-chloride media. Parts A and B were carried out in a medium of 100 mM KCl and 2 mM Tris-succinate; C and D in 100 mM NaCl and 2 mM Tris-succinate; E and F in 50 mM NaCl, 50 mM KCl, and 2 mM Tris-succinate. The concentration of gramicidin was 2×10^{-6} M; valinomycin 2.5×10^{-7} M.

Discussion

The use of osmotic swelling of mitochondria suspended in simple isotonic salt solutions to evaluate the permeability of the mitochondrial membrane to anions and cations was initiated by Chappell and Crofts (1966) and has been extended in a number of laboratories (Henderson *et al.*, 1969; Azzi and Azzone, 1967a,b; Mitchell and Moyle, 1969b; Brierley *et al.*, 1968; Hunter *et al.*, 1969). These studies, in agreement with direct determination of the Cl^- content of isolated pellets of mitochondria (*cf.* Tarr and Gamble, 1966, or Hunter and Brierley, 1969, for example), have established that the inner membrane of the heart mitochondrion is not permeable to Cl^- under a number of metabolic conditions. This limited permeability to Cl^- is apparently not a property of mitochondria from all sources, since corn mitochondria (Wilson *et al.*, 1969) seem to be freely permeable to this anion. As mentioned previously, Azzi and Azzone (1967a) have established that the permeability of mitochondria to Cl^- can be increased by increasing the pH of the suspending medium. In addition, it has been reported that permeability to Cl^- can be induced following modification of the mitochondrial membrane by interaction with Zn^{2+} and other divalent cations (Brierley *et al.*, 1968) and with mercurial reagents under certain conditions (Knight *et al.*, 1968).

The present studies establish that respiring heart mitochondria suspended in 100 mM KCl undergo a cycle of osmotic swelling followed spontaneously by an energy-linked contraction when treated with a high level of valinomycin or gramicidin. Since these particles appear to be impermeable to Cl^- in the presence of respiration or when valinomycin is added to mitochondria in the absence of a source of energy at pH 7.0, it has been suggested (Brierley, 1969) that valinomycin and K^+ induce an energy-linked alteration in the permeability of the mitochondrion under these conditions. The energy-linked osmotic swelling at pH 7.0 reported in the present study is accompanied by an extensive, but transient, acidification of the medium. The possibility, therefore, arises that a corresponding pH increase in the coupling membrane or the matrix is generated by the conditions imposed (high valinomycin, respiration, and a suspending medium of KCl alone). The possibility that this energy-linked pH increase could mimic the effects of a suspending medium of high pH on the permeability of the membrane to Cl^- was therefore examined and will be developed in some detail in the following discussion.

Any model for the explanation of the cyclic reaction observed in 100 mM KCl at pH 7 in the present study must account for the known ability of valinomycin to complex K^+ and carry it across a number of natural and artificial lipid barriers (Pressman *et al.*, 1967) and for previously reported proton shifts in mitochondria (for references, see Greville, 1969, or Packer and Utsumi, 1969) and for the following experimental observations: (a) Initiation of the rapid swelling (Cl^- uptake) phase of the reaction requires a source of energy in addition to a high level of valinomycin. (b) The contraction phase of the reaction is also energy dependent and ensues spontaneously without the addition of inducers or other reagents. (c) Respiration is activated by valinomycin and remains at a high rate through both the swelling and contraction cycles. (d) A glass electrode in the suspending medium records a rapid cycle of acidification followed by a spontaneous reversal to nearly the initial pH. This pH cycle is completed during the swelling phase of the reaction. (e) The glass electrode shows that net pH shifts do not occur during the contraction phase, but that an alkaline shift occurs on anaerobiosis or upon addition of an inhibitor or uncoupler.

In addition to these observations the osmotic swelling responses obtained with beef heart mitochondria in various suspending media shown in the present work and in previous studies (Brierley *et al.*, 1968) must be integrated into the model. A number of these swelling results are summarized in Table II. Swelling and contraction of mitochondria suspended in NH_4Cl are discussed in the accompanying communication (Brierley and Stoner, 1970).

Of a number of models presently available in the literature (Mitchell, 1966; Van Dam and Slater, 1967; Harris and Pressman, 1969; Blondin *et al.*, 1969; Rasmussen *et al.*, 1965;

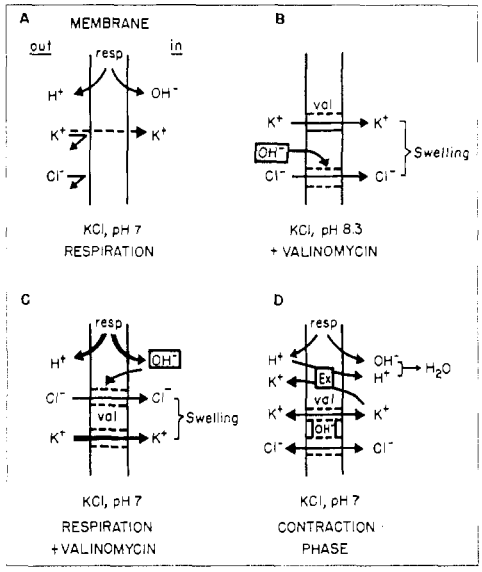

FIGURE 13: Proposed model for osmotic swelling of mitochondria in 0.1 M KCl under the indicated conditions. See the text for explanation.

for example) the chemiosmotic coupling hypothesis of Mitchell appears to be most useful in explaining the existing data on osmotic swelling and contraction. This model (Mitchell, 1966; Mitchell and Moyle, 1969a,b; Greville, 1969) proposes that an anisotropic electron transport system ejects protons on the outside of the coupling membrane of the mitochondrion and that through a system of exchange diffusion carriers this proton gradient is reflected in a membrane potential of about 250 mV with the interior negative.

In terms of such a model the osmotic swelling of mitochondria in isotonic KCl could be explained as follows: at pH 7.0, in the presence or absence of respiration, K^+ permeability is limited and Cl^- penetration is virtually absent (Figure 13A). Respiration is limited by the negative interior potential and excess K^+ which enters the matrix compartment (inside in the diagrams of Figures 13 and 14) is exchanged by a K^+ for H^+ exchange diffusion carrier in the membrane. In-

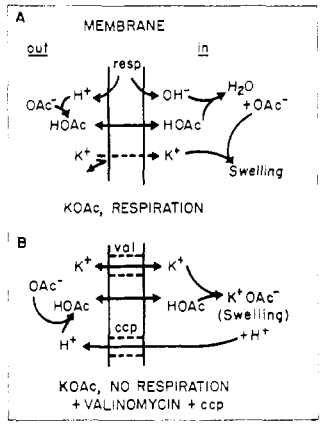

FIGURE 14: Model for osmotic swelling of mitochondria in 0.1 M K^+-acetate.

TABLE II: Summary of Osmotic Swelling by Isolated Heart Mitochondria in Various Suspending Media.

Medium	pH	Conditions	Swelling Response
KCl (100 mM)	7	No energy	No swelling
		Energy	No swelling
		Val, no energy	No swelling
		Val, energy	Swelling, then contraction
		Val and CCP	No swelling[a]
KCl	8.3	No energy	No swelling
		Energy	No swelling
		Val, no energy	Swelling
		Val, energy	Swelling, then contraction
		Val, CCP	Swelling
KOAc (100 mM)	7	No energy	No swelling
		Energy	Swelling
		Val, no energy	Slow swelling
		Val and energy	Rapid swelling
		Val and CCP	Rapid swelling
NH_4OAc	7	All conditions	Rapid swelling

[a] Observed swelling will depend on order of addition (*cf.* Figures 4 and 7).

creasing the pH causes the membrane to become permeable to Cl^- (Figure 13B) and since mitochondria contain little of this anion, the chemical gradient will favor entrance of Cl^- into the matrix and a redistribution of components in accord with the Donnan equilibrium. Net ion uptake and swelling are still limited by K^+ permeability, however, until valinomycin is also added. At high pH and with valinomycin present a rapid passive osmotic swelling is observed (Azzi and Azzone, 1967a), since both anion and cation are now free to move across the membrane in the direction of their chemical gradients (Figure 13B). The movement of the K^+–valinomycin complex can be visualized as essentially an electrophoretic response which permits positive charges to flow into the negative interior. The strong dependence of the Cl^- permeability of the membrane on pH suggests that energy-linked pH changes may be responsible for at least the initial phases of the respiration-dependent osmotic swelling observed at pH 7 when valinomycin is added (Figure 13C). In terms of the present model the combination of high concentration of K^+ and valinomycin would result in a massive surge of K^+ into the matrix space which would decrease the net negative charge and result in respiration-dependent H^+ and OH^- production on the opposite sides of the membrane (Figure 13C). In support of this suggestion Mitchell *et al.* (1968) have noted an apparent transient alkalinization of the mitochondrial membrane using the bromothymol blue technique following a pulse of oxygen in valinomycin-treated mitochondria suspended in 0.15 M KCl. These authors have also pointed out some of the difficulties in interpretation of bromothymol blue responses, however. More recently Harris (1969b) has shown an increase in intramitochondrial pH by both the bromothymol blue technique and distribution of

dimethylisoxazolidinedione following initiation of K^+ uptake by valinomycin.

The locally high pH which thus results could be considered as the vector which permits increased permeability to Cl^- in much the same way as shown for the externally applied pH increase (Figure 13B). Cl^- would now flow into the matrix space and osmotic swelling would commence. The resulting influx of KCl (which appears to occur passively at elevated pH and therefore must be considered to be in the direction of the net chemical and electrical gradients (*cf.* Rossi and Azzone, 1969)) establishes a new condition in the matrix which is characterized by a high concentration of K^+ and Cl^-. The observed reversal of the pH gradient and the energy-linked contraction which follow (Figure 1) can be explained in this model by the action of the postulated H^+/cation antiport (Mitchell, 1966) or exchange diffusion carrier. When sufficient K^+ is present in the interior, the exchange of external H^+ for internal K^+ ("Ex" in Figure 13D) results in an influx of H^+ into the interior from the suspending medium and reversal of the glass electrode trace. Since the entering H^+ reacts with an OH^- of the interior there is a net efflux of positive charge under these conditions and if Cl^- were to shift out in response to this exchange, a net respiration-dependent osmotic contraction would result. The rate and extent of contraction will depend on whether the exchange reaction can exceed the influx of K^+ under the influence of valinomycin and Cl^- under the influence of the high pH in or in the vicinity of the membrane. It should be noted that the influx of H^+ in exchange for K^+ would result in a net decrease in interior pH (restricting Cl^- entrance) and a net decrease in negative charge in the interior (restricting the passive influx of K^+). When respiration ceases at anaerobiosis, or when an inhibitor or uncoupler is added, the existing pH gradients equilibrate and the permeability of the membrane to Cl^- is now reversed to its initial low value.

These considerations would account for the osmotic responses shown in Figure 9 which indicate that in the absence of respiration the membrane is not permeable to Cl^- whereas in the presence of respiration during the swelling phase there is considerable permeability to this anion and during the contraction phase an intermediate condition appears to prevail. The fact that energy-linked contraction can be obtained in a suspending medium of pH 8.3 (Figure 6) indicates that the pH-dependent Cl^- permeability is not the predominant factor in whether this phase of the reaction can occur, but the decreased extent of contraction under these conditions suggests that Cl^- permeability has some influence in the overall cycle observed. Azzi and Azzone (1967b) have documented the contraction phase of the cyclic reaction presently under discussion and found it to have a pH optimum of about 7.5.

The sequence of events at low temperature (Figure 7) supports the model just described (Figure 13) except for the fact that considerable swelling occurs after the discharge of the pH gradient by addition of CCP. In this study little swelling occurs until after the H^+ shift has reached rather large proportions and discharge of the gradient with CCP during this phase of the reaction inhibits the swelling. In the absence of CCP the low rate of respiration at this temperature might result in the production of H^+ at a rate sufficient to exchange with internal K^+ and neutralize the pH gradient (as in Figure 13D) but not produce sufficient H^+ to enter and neutralize the internal OH^-. At 10° this does not immediately stop the osmotic swelling and Cl^- uptake, however. It appears possible that additional transient permeability effects of the uncoupler (*cf.* Caswell and Pressman, 1968; Caswell, 1969) may predominate under these conditions, or that a further time factor in equilibrating a portion of the OH^- at 10° may be involved.

Several other lines of evidence also indicate that factors in addition to the internal and external pH may control permeability and swelling under some conditions. For example, the qualitative difference in response to low levels of valinomycin (which produce large pH shifts, as seen in Figure 2) and to the high concentrations of the antibiotic necessary to elicit maximum swelling hints that the rate of OH^- production may be the controlling feature for the initiation of swelling. Alternatively, it could be suggested that a portion of the swelling is due to some action of high levels of valinomycin which is not related to the pH shifts. Studies with aged mitochondria indicate that the swelling elicited by valinomycin under the conditions of Figure 1 consists of two phases, one closely related to the H^+ production and sensitive to uncouplers, and a second more rapid phase of swelling which is largely insensitive to uncouplers and commences when the extrusion of H^+ ceases and the pH trace is reversing. This response is similar to that seen at low temperature (Figure 7) and is consistent with the suggestion that a general collapse of membrane permeability properties is associated with the swelling which accompanies the reuptake of extruded protons. Experiments with passive swelling at high pH (manuscript in preparation) indicate that once a high pH is imposed on a suspension of mitochondria in the absence of respiration and the rapid swelling has been initiated by valinomycin, a pulse of acid which reestablishes a neutral pH fails to stop the swelling immediately. Time (1–2 min) is required for the low rate of swelling to be established following this treatment. This result, like those presented in Figure 7 for the respiring system at low temperature, indicates that once the permeable (swelling) condition has been well established, it is not under the immediate, direct control of pH.

The alteration in membrane permeability induced under the conditions of Figure 1 is not specific for Cl^-. Br^-, NO_3^-, and I^- give the same response (Figure 11) and in addition, such substrate anions as succinate, fumarate, and citrate support the osmotic swelling. Since fumarate normally has the properties of a nonperme antanion (Chappell and Haarhoff, 1967), this result suggests that the specific carrier mechanisms which have been proposed to account for observed anion penetration do not participate in the present reactions, and that the swelling supported by these anions in the present study results from a nonspecific permeability change. Such a pathway would correspond to the electrogenic "pathway B" discussed by Azzone and Piemonte (1969). It should also be noted that numerous studies in the literature have indicated that the energy status and K^+ flux of the mitochondria are closely related to the uptake of anions (see Slater, 1969, Harris, 1969a, Van Dam and Tsou, 1969, Pressman, 1969, Haslam and Griffiths, 1968, Gamble, 1965, Quagliariello and Palmeri, 1969, Max and Purvis, 1965, and Ferguson and Williams, 1966, for discussion and additional references).

Addition of a permeant anion such as acetate or phosphate results in a different set of osmotic responses (Table II; Figures 10 and 11). The model of Figure 13 can also be used to explain the energy-linked swelling seen under these conditions (Figure 14). In the absence of a source of energy (respira-

tion or exogenous ATP) K^+ fails to cross the membrane due to its intrinsic low permeability and, while acetate can penetrate the membrane as un-ionized acetic acid, no swelling can occur in the absence of a penetrating cation. In the presence of respiration, acetic acid functions as a H^+ carrier to neutralize excess OH^- in the internal compartment (in much the same way as many investigators envision an uncoupler would act) and stimulates respiration. Regardless of whether the membrane permeability to K^+ is altered by respiration (a possibility) or whether the electrogenic effects of the buildup of OAc^- in the interior predominate, the net effect is a rapid energy-linked uptake of K^+-acetate and osmotic swelling in this medium. Addition of valinomycin enhances K^+ permeability and accelerates the energy-linked ion uptake and swelling. In the absence of energy valinomycin induces a slow swelling of the mitochondria in isotonic KOAc (Figure 14B). This swelling is accelerated by addition of an uncoupler. In keeping with a recent suggestion by Henderson *et al.* (1969) the uncoupler may act by permitting H^+ to exit from the interior and permit rapid K^+ and OAc^- buildup as shown in the diagram. Permeant anions such as acetate and phosphate would not support contraction in this model since the H^+ for K^+ exchange carrier is circumvented by the rapid influx of protons with the entering acetic acid.

In conclusion it is apparent from the present results and those of the companion study (Brierley and Stoner, 1970) that the postulates of the chemiosmotic coupling hypothesis of Mitchell (1966) can be used to explain a large number of osmotic swelling and contraction responses of mitochondria suspended in simple salt media. The present work offers the possibility that under certain circumstances the membrane potential can be reflected in a pH gradient which controls the permeability of the membrane to anions. Conditions in which a major portion of the membrane potential can be reflected as a pH gradient have been examined by Mitchell and Moyle (1969a). Other models currently under consideration can also be used to explain the existing data, but most appear to require additional *ad hoc* assumptions. A number of examples of energy-linked contractions of swollen mitochondria are available in the literature (Lehninger, 1962; Wilson *et al.*, 1969; Blair and Sollars, 1967; Crofts and Chappell, 1965; Azzi and Azzone, 1967b; Knight *et al.*, 1968). The H^+ for K^+ exchange diffusion system offers a mechanism for the phenomenon of energy-linked contraction which does not require additional assumptions, such as the existence of an actomyosin-like contractile component, an outwardly directed pump system for anions or cations or a major reorientation of the existing membrane.

The existence of such a contraction mechanism in the case of simple suspending media as documented here brings up the question of whether both the swelling and contraction phases of oscillating volume changes of mitochondria in more complicated media (Chance and Yoshioka, 1966; Packer *et al.*, 1966; Höfer and Pressman, 1966; Graven *et al.*, 1966; Falcone and Hadler, 1968) may be energy-linked and under the influence of membrane pH changes. In many of these cases the contraction phase appears to be passive, but the presence of sucrose in the suspending medium superimposes an osmotic contraction which would make the presence of an energy-linked component difficult to detect.

These osmotic swelling studies shed no additional light on the question of whether the membrane potential is the driving force for ATP synthesis (Mitchell, 1966). The swelling and contraction seen in the present work can be supported by exogenous ATP in the same way as by respiration, however, a result which indicates that the two systems can be directed into the same swelling and contraction events in the membrane and are in close communication.

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