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Energetics of Potassium Transport in Mitochondria Induced by Valinomycin*

Ronald S. Cockrell,† Eric J. Harris,‡ and Berton C. Pressman§

ABSTRACT: The valinomycin-induced transport of K^+ into mitochondria has been followed on a continuous basis, by means of the ion-specific electrode, as a function of [K^+], pH, temperature, energy source, and anionic environment. Simultaneous measurement of O_2 consumption during transport energized by substrate oxidation, or liberation of inorganic phosphate (P_i) during ATP-energized transport, yields values for the stoichiometry of K^+ transport per equivalent of adenosine triphosphate (ATP) expended. A maximum value over seven was obtained at pH 6.7, 25°, 2.5 mM K^+, and 20 mM acetate for ATP-energized transport, corresponding to a thermodynamic efficiency estimated to be about 80%.

The energy of activation of K^+ transport obtained from the temperature studies was 9.8 and 10.8 kcal for oxidizable substrate and ATP-energized transport, respectively. Evidence is offered that the mechanism of action of valinomycin involves not only increased mitochondrial permeability to K^+ but also stimulation of the transport-energizing process itself. The data obtained have been used for a critical assessment of proposed mechanisms of ion transport, particularly the chemiosmotic hypothesis of Mitchell.

The accumulation of K^+ by mitochondria, induced by valinomycin (Moore and Pressman, 1964), shares in common with the spontaneous, energy-dependent accumulation of the divalent ions, Ca^{2+},[1] Mn^{2+} (Maynard and Cotzias, 1955; Bartley and Amoore, 1958; Saris, 1963; Chappell *et al.*, 1963), Sr^{2+} (Saris, 1963; Chappell *et al.*, 1963; Carafoli, 1965), and Mg^{2+} (Brierley, 1963; Pressman and Park, 1963; Judah *et al.*, 1965a): (1) a requirement for energy from either oxidizable substrate or ATP;[2] (2) sensitivity to specific inhibitors, such as oligomycin (energy source, ATP), and to amytal or rotenone (energy source, oxidizable substrate); (3) stimulated respiration or ATPase activity; (4) movement of H^+ counter to movement of ion accumulated; and (5) facilitation by permeant anions, such as phosphate, arsenate, and acetate (Moore and Pressman, 1964; Rasmussen *et al.*, 1964; Pressman, 1965a; Chance and Yoshioka, 1965; Rasmussen *et al.*, 1965). This suggests that the underlying mechanisms of mono- and divalent ion accumulations are similar if not identical. Indeed, a mutual competition between monovalent and divalent cation accumulation (guanidinium and Mg^{2+}) has already been reported (Pressman and Park, 1963). Accordingly, information obtained for any one cation might be expected to apply generally to the phenomenon of energy-dependent cation accumulation by mitochondria.

The induced accumulation of K^+ has definite techni-

* From the Department of Biophysics and Physical Biochemistry, Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania. *Received April 11, 1966.* This investigation was supported by a U. S. Public Health Service grant (GM 12202) from the Institute of General Medical Sciences, the U. S. Public Health Service.

† Trainee, National Institutes of Health training grant (ST 1-GM-694-05).

‡ Visiting Lecturer at the Johnson Foundation, 1966. Permanent address, Department of Biophysics, University College, London, England.

§ Recipient of a U. S. Public Health Career Development Award (K 3-GM-3626).

[1] The rather extensive bibliography pertaining to the interaction of Ca^{2+} with mitochondria is reviewed exhaustively in the recent paper by Chance (1965).

[2] Abbreviations used: ADP and ATP, adenosine di- and triphosphate; DPN, diphosphopyridine nucleotide.

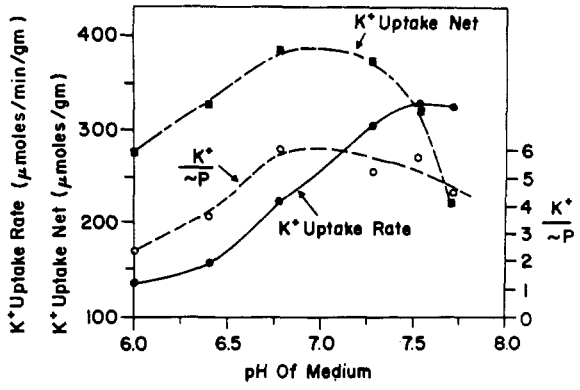

FIGURE 1: Effect of pH on potassium accumulation energized by ATP. Reactions were initiated by the addition of 0.2 μg of valinomycin to 12 ml of reaction mixture containing: 40 mM Tris acetate, 20 mM Tris chloride, 5 mM KCl, 5 mM ATP, 12 μg of rotenone, 250 mM sucrose, and approximately 30 mg of mitochondrial protein, $T = 25°$. For each determination the reaction was adjusted to the required pH with HCl or Tris base.

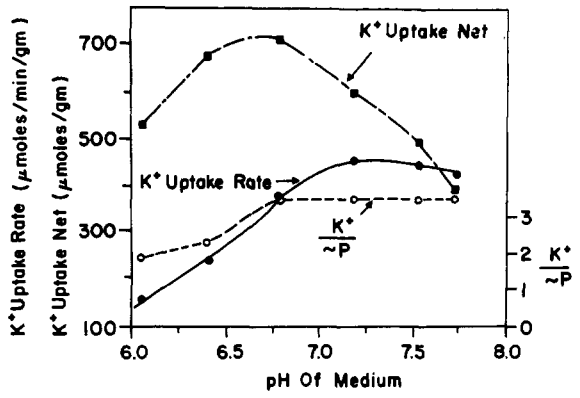

FIGURE 2: Effect of pH on potassium accumulation energized by oxidizible substrate. Reaction conditions were the same as in Figure 1 except for the substitution of 2.5 mM glutamate and 2.5 mM malate for the ATP and rotenone.

cal advantages for study over divalent ion accumulation notably the amenability of the process to continuous monitoring by ion-specific electrodes (Moore and Pressman, 1964), the minimal deleterious effects of higher concentrations (*cf.* uncoupling and lysis by Ca^{2+}), and the freedom from complications arising from secondary binding and/or precipitation (*cf.* Ca^{2+} with P_i and organic phosphates). It is also convenient to be able to initiate the process precisely at will by the addition of catalytic amounts of valinomycin and certain other antibiotics.

The observed stoichiometry of about two Ca^{2+} accumulated per equivalent ~P[3] expended has given rise to speculations on the mechanism of the accumulation of ions by mitochondria (Rasmussen *et al.*, 1965; Chance, 1965). A generally applicable mechanism should also accommodate the analogous $K^+/\sim P$ ratios, already reported as high as 3.6 with oxidizable substrate (Pressman, 1965a). The present communication extends these $K^+/\sim P$ determinations, highly definitive for evaluating proposed mechanisms of mitochondrial ion transport, to the ATP-energized system. The effects of pH, temperature, anion species, and extramitochondrial $[K^+]$ on the rate and extent of net transport, as well as the $K^+/\sim P$ stoichiometry, have also been compared with either ATP or oxidizable substrate as energy sources.

Methods and Materials

Rat liver mitochondria were isolated essentially as described by Schneider (1948), homogenized in 0.25 M sucrose–1 mM EDTA (pH 7.4), thrice washed in 0.25 M sucrose, and used within 1 hr of preparation. K^+ accumulation and pH were monitored by means of Beckman 39047 and A. H. Thomas 4858 combination electrodes, respectively; O_2 consumption was followed with a Radiometer E-5044 Clark-type electrode. All electrode outputs were recorded simultaneously by means of an oscillographic recorder. A brief description of the apparatus and calibration techniques has already appeared (Pressman, 1965a); a more detailed account of the apparatus and calibration procedures will be presented elsewhere (Pressman, 1966). ATPase activities were obtained from the rate of P_i liberation as determined from periodically withdrawn aliquots by the procedure of Wähler and Wollenberger (1958) following fixation in cold 6% $HClO_4$.

The steady-state level of ADP attained in various systems was determined by incubating mitochondria (5 mg of equivalent protein) on a shaking bath (25°) in the following basic medium: 20 mM Tris chloride, 25 mM KCl, 0.2 mM EDTA, 2.0 mM P_i, 5 mM ATP, and 250 mM sucrose, final pH 7.8, final volume, 3.0 ml. After 5 min the reaction mixtures were deproteinized with $HClO_4$ (final concentration, 0.5 M) and a cleared aliquot brought to pH 6.5 with a mixture containing 2 M KOH, 1.35 M triethanolamine, and 50 mM acetic acid. Aliquots were then analyzed fluorometrically for ADP by the method of Maitra and Estabrook (1964). In certain experiments the mitochondria were pretreated with rotenone (140 μg/g of equivalent mitochondrial protein). Protein was determined by the biuret method as described by Layne (1952).

Results

Figures 1 and 2 compare the rates and net accumulation of K^+ induced by valinomycin as a function of pH for both ATP- (+ rotenone) supported transport

[3] Although the designation "~P" commonly represents a high energy phosphate group such as the terminal one of ATP, in this paper it also represents the equivalent energy quanta arising from the oxidation of the glutamate-malate substrate system assuming three ~P per atom of O consumed.

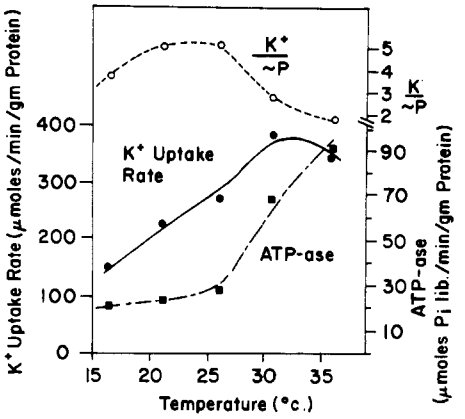

FIGURE 3: Effect of temperature on potassium accumulation energized by ATP. Conditions were the same as in Figure 1 except for the fixed pH, 6.7, and the variable temperature.

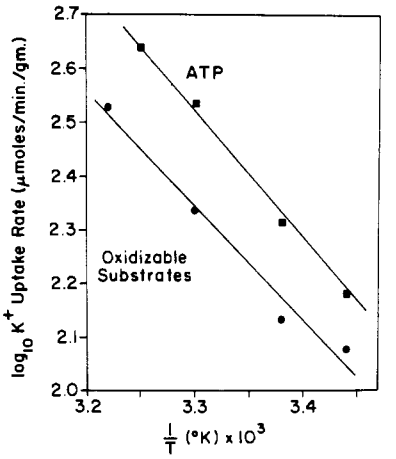

FIGURE 4: Arrhenius plot of the effect of temperature on potassium accumulation. Conditions were the same as in Figure 1 for ATP driven accumulation and Figure 2 for oxidizable substrate driven accumulation. Suboptimal amounts of valinomycin (0.1 μg) were added to the oxidizable substrate driven system in order to bring its rate into line with the ATP driven system. Temperatures were varied as indicated and the pH was maintained at 6.7.

(Figure 1) and transport supported by glutamate–malate (Figure 2). Rotenone was used to suppress energy production by oxidation of endogenous substrate. Although it inhibits only DPN-linked respiration, and not that due to succinate (Ernster *et al.*, 1963), rotenone was preferred over the more universal inhibitor antimycin (Potter and Reif, 1952), since the latter also affects energy transfer as evidenced by stimulation of ATPase (Maley and Johnson, 1957). $K^+/\sim P$ ratios have been calculated on the basis of the total observed respiratory and ATPase *rates*, without subtracting the base rates before the addition of valinomycin, and the initial rates of K^+ accumulation which, in the presence of 40 mM acetate, remained reasonably linear for at least 30 sec. Since it is unlikely that the base rates of energy expenditure could be diverted entirely to support ion transport following valinomycin addition, the $K^+/\sim P$ ratios calculated must be regarded as minimal.

The effect of pH on the rate of induced K^+ accumulation was compared with either ATP or oxidizable substrate as energy source. With ATP, both net K^+ uptake and $K^+/\sim P$ are maximal at pH 7.0. Maximal K^+ uptake rates occur at a somewhat higher pH (Figure 1). On the other hand, with oxidizable substrate as energy source, both net K^+ uptake and the K^+ uptake rate are maximal at a somewhat lower pH, and nearly twice as great as in the ATP system, although the $K^+/\sim P$ is lower (Figure 2). The K^+ uptake rates with oxidizable substrate exceed those obtained in the ATP system, particularly at lower pH.

The effect of temperature on the rate of K^+ accumulation was also compared for different energy sources. The Q_{10} values of both systems are temperature dependent, in agreement with the report by Azzi and Azzone (1965) for valinomycin-induced K^+ transport. Over the interval 20–30° the Q_{10} values found by us with ATP and glutamate–malate are 1.8 and 2.0, respectively. The K^+ uptake rates decline as the temperature is raised above 30°.

Temperature also exerts a profound influence on the $K^+/\sim P$ ratio. The rate of K^+ uptake and ATPase, along with the derived $K^+/\sim P$ ratios, as functions of temperature, are shown in Figure 3. As the temperature increases, energy dissipation, as reflected by the hydrolysis of ATP, rises more slowly than the K^+ uptake rate until 25°, at which temperature the maximum $K^+/\sim P$ is reached. The decline in efficiency as the temperature increases above 25° is rapid, indicating that measurements at room temperature require strict temperature control for reproducibility.

An Arrhenius plot of the data used to calculate the Q_{10} values (Figure 4) gives energies of activation of 9.8 kcal/mole of K^+ for K^+ accumulation supported by oxidation of substrate and 10.8 kcal/mole of K^+ when supported by ATP. This implies that the rate-limiting reaction in K^+ accumulation supported with either ATP or oxidizable substrate is probably identical (within the range of our experimental error), so that the different $K^+/\sim P$ ratios indicate that coupling of a common hypothetical energized intermediate, "$\sim$," is "tighter" with ATP than with the electron transport chain. These energy of activation figures are appreciably higher than values for Na^+ and K^+ movement by simple diffusion, 4.5 kcal (Solomon, 1952), and typical of other transport processes such as the turnover of K^+ in erythrocytes, 12 kcal, Na^+ turnover in erythrocytes, 20 kcal, influx of glucose in erythrocytes, 13 kcal (Solomon, 1952), and the entry of SO_4^{2-} into mitochondria, 12 kcal (Davies *et al.*, 1960). From this we infer that the

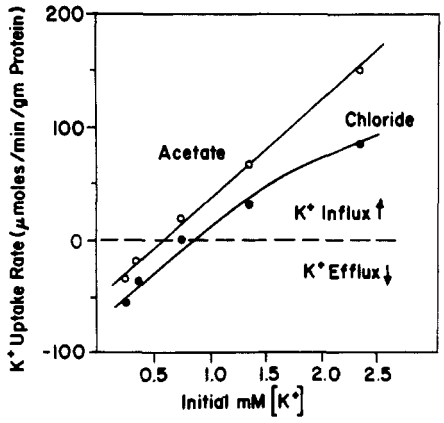

FIGURE 5: Effect of extramitochondrial potassium concentration on the rate of ATP-energized potassium accumulation with and without added acetate. Conditions were the same as in Figure 1 except for the omission of Tris acetate in the chloride series and maintenance of pH at 6.7.

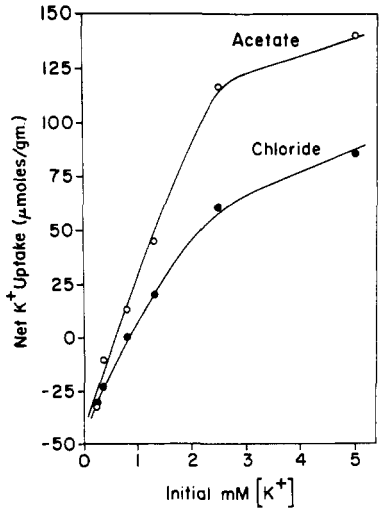

FIGURE 6: Effect of extramitochondrial potassium concentration on the net accumulation of potassium with and without added acetate. Conditions were the same as in Figure 5.

ion translocation process induced by valinomycin is not likely to be limited by diffusion but rather by reactions more closely associated with energy transduction.

The influence of external [K^+] and anionic environment on ATP-supported K^+ uptake is shown in Figures 5–7. Valinomycin causes a net efflux of K^+ below 0.8 mM K^+ in the absence of acetate, below 0.6 mM K^+ in its presence (Figures 5 and 6). Between 0.6 and 0.8 mM K^+, therefore, acetate accounts for the qualitative difference between K^+ influx and K^+ efflux. The beneficial effects of acetate on K^+ uptake rate and the net K^+ accumulation are considerably greater at higher K^+ concentrations, as has been reported elsewhere (Harris *et al.*, 1966). If rotenone is omitted, K^+ influx occurs when valinomycin is added, even from the lowest K^+ concentrations and with no added substrate, indicating that whatever exogenous oxidizable substrate is present is a more potent, if less efficient, energy source for K^+ accumulation than is exogenous ATP.

The $K^+/\sim P$ ratio obtained in the presence of ATP rises from negative values at low extramitochondrial [K^+] to a maximum of 7.9 at 2.5 mM K^+. The negative values indicate that, despite the increased energy expenditure associated with elevated ATPase, under these conditions valinomycin induced a net K^+ efflux. The energy liberated by the increased ATPase activity in such cases is presumably dissipated as a higher flux of K^+ across the mitochondrial membrane; since the gross efflux exceeds the gross influx, no net K^+ influx is observed. The net K^+ efflux from mitochondria initiated by valinomycin at low extramitochondrial [K^+], where the energy requirements for net uptake can exceed the availability of energy from ATP, is a key observation which any mechanism for induced ion transport must explain. The rise in $K^+/\sim P$ as the [K^+] is elevated, seen in Figure 7, presumably reflects the decreasing free energy requirements for K^+ transport as the opposing gradient is reduced by raising the extramitochondrial [K^+]. At still higher [K^+], decreased $K^+/\sim P$ ratios are obtained. Under these conditions some type of inhibitory process occurs which is manifested as a rapid decay of the initially rapid K^+ uptake rates. This same inhibitory process also gives rise to various oscillatory phenomena characteristic of induced ion transport under certain conditions. The inhibition is reduced by acetate which, particularly at higher [K^+], increases the *rate* of K^+ accumulation, the *net* K^+ accumulated, and the $K^+/\sim P$ ratio over those values obtained in the acetate-free medium.

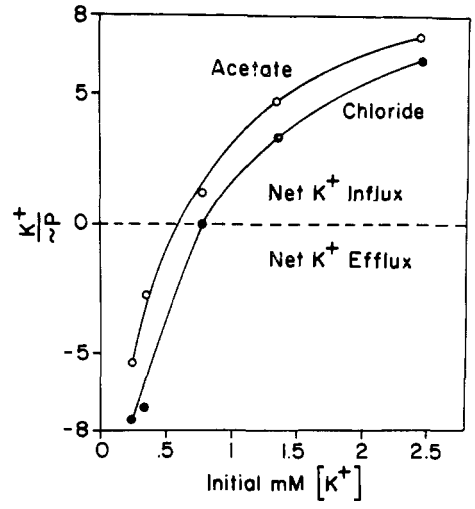

FIGURE 7: Effect of extramitochondrial potassium concentration on the $K^+/\sim P$ ratio with and without added acetate. Conditions were the same as in Figure 5.

TABLE I: Steady-State Levels of ADP in Mitochondrial Systems.

Additions (mM)	Treatment of Mitochondria by Rotenone	ADP Present after Incubation: Control (μM)	ADP Present after Incubation: 280 μM ADP Initially Added	ADP Present after Incubation: Added ADP Recovd
None	−	140	310	170
None	+	160	425	265
Succinate (3)	−	90	95	5
Succinate (3)	+	100	105	5
Succinate (3) + 30 μg of oligomycin	+	130	350	220
β-Hydroxybutyrate (6)	−	130	125	−5

Factors expected to affect the $K^+/\sim P$ ratio include the quality of a given mitochondrial preparation, pH, temperature, and the ratio of extramitochondrial $[K^+]$ to the internal K^+ concentration, *i.e.*, the K^+ gradient. Under optimal conditions (pH about 7.0, $[K^+]$ = 2.5–7.5 mM) a series of seven experiments gave an average $K^+/\sim P$ ratio of 6.5 ± 0.15 (std error). The relatively high $K^+/\sim P$ ratios encountered, and their great dependence upon reaction conditions, suggest that the mechanism of ion accumulation does not involve a simple fixed integral $K^+/\sim P$ ratio paralleled by the integral $\sim P/O$ ratios usually obtained when measuring oxidative phosphorylation.

A particular point, raised in the discussion below, required an experimental determination of the chemical potential against which mitochondria can synthesize ATP. Mitochondria were incubated with ATP under the conditions listed in Table I, and, after reaching the steady state, the ADP present was determined. In expt 1, endogenous substrate alone drove the level of ADP, initially present chiefly as a contaminant of the commercial ATP, down to 140 μM. In a parallel experiment, the introduction initially of a 280 μM ADP supplement led to a recovery of ADP at a level 170 μM above the control, indicating that part of the added ADP was phosphorylated. When rotenone-treated mitochondria were used (expt 2), 160 μM ADP was recovered. This presumably is the sum of the ADP originally present in the ATP plus a small amount bound to the mitochondria. Recovery of a 280 μM supplement added to this system was virtually complete. Addition of succinate as substrate (expt 3) drove the ADP down to 90 mM, both with and without the ADP supplement, establishing that this value represents a true steady-state level. Comparable figures were obtained with rotenone-treated mitochondria (expt 4); since this system cannot oxidize succinate beyond fumarate, substrate level phosphorylation is not involved in determining the steady-state level of ADP. Phosphorylation of the ADP supplement was inhibited by oligomycin (expt 5), removing all doubt that the disappearance of added ADP results from oxidative phosphorylation. A somewhat higher steady-state level of ADP, 130 μM., was obtained for the one-step oxidation of β-hydroxybutyrate (expt 6), indicating that the steady state is subject to differences in the energy potential of the substrate system present.

Discussion

The maximal $K^+/\sim P$ ratios reported here, 3.2 for oxidizable substrate and over 7 for ATP-supported ion accumulation, are higher than the corresponding values previously reported for mitochondrial divalent ion accumulation. The ratios supported by ATP exceed those obtained for Ca^{2+}, even after dividing by two to allow for the difference in charge between K^+ and Ca^{2+}. Values of 1.8 for $Mn^{2+}/\sim P$ (Chappell *et al.*, 1963), 1.8–2.1 for $Sr^{2+}/\sim P$ (Carafoli, 1965), and $Ca^{2+}/\sim P$ values approaching two have been reported (Chance, 1956; Rossi and Lehninger, 1964; Rasmussen *et al.*, 1965; Chance, 1965), although recent work indicates that under alkaline conditions the $Ca^{2+}/\sim P$ supported by oxidizable substrate is quite variable, attaining values as high as five to ten (Carafoli *et al.*, 1965), comparable to our figures with K^+. This may be due in part to the lowering of the association product of the various calcium phosphate complexes and precipitates. In our experiments, raising the pH above 7 does not raise the $K^+/\sim P$ ratio. The low $Ca^{2+}/\sim P$ ratios obtained with ATP as an energy source (Rossi and Lehninger, 1964) could be due to a direct activation of ATPase by Ca^{2+} not related to ion translocation and, therefore, are not necessarily valid indications of the true stoichiometry determined by the mechanism of ion transport.

The reported values for mitochondrial K^+ content range from 115 to 165 μmoles/g of protein (Bartley and Davies, 1954; Gamble, 1957; Berger 1957). We have observed values of approximately 100 μmoles/g of protein for our preparations. If we assume the K^+ to be dissolved in an intramitochondrial water space

of 2.5 ml/g of protein (Bartley and Davies, 1954), the calculated initial intramitochondrial [K^+] is 40 mM, in good agreement with Werkheiser and Bartley (1956). During induced K^+ uptake, the mitochondrial volume increases, keeping pace with the influx of K^+ so that the intramitochondrial [K^+] is more or less maintained at approximately 40 mM. A somewhat higher value, 140 milliosmolar, equivalent to 70 mM K^+, was given in our previous paper (Harris *et al.*, 1966), but estimation of the mitochondrial water space was deliberately conservative. Values greater than our best estimates, 40–50 mM K^+, would further increase the energy required for K^+ accumulation and raise the apparent free energy of the process.[4] Since the [K^+] of the medium did not decrease appreciably from its initial value of 2.5 mM, the concentration term of the free energy necessary to transport K^+ against the 16:1 concentration gradient, ΔF_K, can be calculated from the equation

$$\Delta F_K = 2.3\ RT \log \frac{[K^+]_1}{[K^+]_2} = 1.64 \text{ kcal/mole of } K^+ \quad (1)$$

Only a minuscule amount of K^+ could be transported across the mitochondrial membrane before evoking a compensatory movement of other ions in order to preserve electrostatic balance. This balance can be achieved either by moving anions along with the K^+ or eliciting the countermovement of another cation from the mitochondria. In the absence of adequate concentrations of permeant anions such as acetate or phosphate (Rasmussen *et al.*, 1965; Chance and Yoshioka, 1965; Harris *et al.*, 1966), as when chloride alone is the anion, the principal mechanism for neutralizing the charge of the K^+ entering the mitochondria is the outward movement of H^+ (Moore and Pressman, 1964). The H^+ out/K^+ in ratio, under some conditions, can approach unity. Under these circumstances, evaluation of the free energy change in the system as a whole, due to H^+ translocation, would require additional information, presently unavailable, about the intramitochondrial pH and buffer capacity, and the source of the translocated H^+. In the presence of 40 mM acetate, however, the H^+/K^+ ratio falls to very low values, indicating, for the most part, the influx of K^+ is accompanied by an equivalent influx of acetate to preserve the charge balance (Harris *et al.*, 1966). Experimentally, the mitochondria were permitted to equilibrate against 40 mM acetate before the initiation of transport by valinomycin. Since, during the influx of K^+, mitochondrial swelling maintains the internal [K^+] at about 40 mM, the acetate accumulating along with the K^+ is reasonably close to the concentration present extramitochondrially, and its possible translocation down a gradient would not lower the free energy required for the K^+ movement more than 10% from our calculated value. Thus in the experiment depicted in Figure 7, the net free energy required by ion translocation is approximately that required for moving K^+ alone, calculated above as 1.64 kcal/mole. If acetate is unavailable to neutralize the K^+ charges, thus requiring a K^+ for H^+ exchange, the $K^+/\sim P$ ratio falls (Figure 7), indicating that, under the conditions defined, more energy is required to move H^+ out of the mitochondria than acetate in.

Direct assay of the media to which the mitochondria and 5 mM ATP had been added (Figure 7, acetate, 2.5 mM K^+) indicated that 0.16 mM P_i was present at the time of valinomycin addition. With a representative value of 0.2 mM for the ADP present in the ATP, the experimental free energy of hydrolysis of ATP, $\Delta F''$, may be computed initially, using -8.4 kcal/mole for the $\Delta F'$ at pH 7.0 and 25° in the absence of Mg^{2+} (Benzinger *et al.*, 1959)

$$\Delta F'' = \Delta F' - RT \ln \frac{[ATP]}{[ADP][P_i]} = \quad (2)$$

$$-8.4 \text{ kcal/mole} - 7.0 \text{ kcal/mole} = -15.4 \text{ kcal/mole of ATP}$$

Thus, under the initial experimental conditions, the energy available from the hydrolysis of one ATP would be sufficient to transport 15.4/1.64 or 9.4 K^+ against a K^+ gradient of 16:1. Typically no more than 0.2 mM ATP is hydrolyzed during the first minute after initiation of K^+ transport by valinomycin. Substituting the resultant values of ATP, ADP, and P_i into the second term of eq 2, the energy of ATP hydrolysis then becomes -14.5 kcal/mole, which would support a $K^+/\sim P$ ratio of 8.8. Thus the observed $K^+/\sim P$ ratio of 7 (Figure 7, K^+ = 2.5 mM) represents a thermodynamic efficiency of almost 80% during the interval of the first minute.

These calculations assume that the sole source of energy for K^+ translocation was ATP hydrolysis. The 0.2 mM ATP hydrolyzed during the above experiment corresponds to 80 μmoles/g of mitochondrial protein. Compared to this, the upper limit of the "non$\sim$P" energy reserve of mitochondria according to the "ATP jump" data of Eisenhardt and Rosenthal (1964) is <1 μmole/g, and therefore negligible.

Since additional factors in the intramitochondrial environment may alter the calculated relationships of coupled processes extending across the mitochondrial membrane, our calculations are not presumed to be thermodynamically rigorous. To the extent that the values used in our calculations represent the best available, and that the calculations are analogous to those appearing in the literature, our conclusions merit interest. Thus our efficiency figures are comparable to 53% reported for Na^+ transport in the gull salt gland (Chance *et al.*, 1964) and 50% for Ca^{2+} transport by the sarcoplasmic membranes (Hasselbach, 1964).

Our calculations presume that enough sucrose en-

[4] We have found that the increase in mitochondrial volume accompanying valinomycin-induced K^+ uptake is independent of the extramitochondrial osmotic pressure over a considerable range. This supports the assumptions made in the present paper and is incompatible with an osmotic basis of ion-induced mitochondrial swelling. A preliminary account of this work was reported by Pressman (1965b). A more detailed account will be presented elsewhere.

tered the mitochondria along with the K^+ and acetate, so that no appreciable additional osmotic work was done. If less than that required amount of sucrose entered, additional osmotic work would be done and the free energy of ATP hydrolysis conserved in gradients would exceed the 80% calculated from the K^+ movement alone.

Chappell and Crofts (1965) and Rasmussen *et al.* (1965) have assumed, without presenting evidence, that the mitochondrial swelling is due only to the entry of the osmotic water equivalent of the transported ions, rather than several times this volume as we have concluded (Pressman, 1965a; Harris *et al.*, 1965, 1966; see also footnote 3). If the swelling were strictly osmotic, calculation of the free energy of ion movement would require knowledge of the initial size and constituents of the mitochondrial compartment entered by K^+. The intramitochondrial concentrations of accumulated K^+ and acetate, and the corresponding gradients, would be markedly greater than the values used in our calculations, and the free energy of ion movement would probably exceed that available from the hydrolysis of ATP.

While we cannot yet offer a detailed mechanism of ion transport, the present data provide useful criteria for evaluation of any proposed mechanism. Thus, mechanisms which invoke ejection of substrate-derived protons (Robertson, 1960) have difficulty in explaining the high $K^+/\sim P$ ratios reported here, even when modified in a manner such as the "potential divider" suggested by Davies (1961). It is particularly difficult to envisage a metabolic source of H^+ driving K^+ movement in the ATP–rotenone system at pH 6, since the net H^+ evolved per ATP split is below 0.1, yet $K^+/\sim P$ ratios of 2.6 are maintained (Figure 2). Mechanisms dependent on substrate-derived electrons (Lundegaardh, 1945; *cf.* also review by Robertson, 1960) are contraindicated by the ability of ATP to energize ion transport in systems in which electron transport is inhibited by antimycin or rotenone.

The chemiosmotic hypothesis, primarily advanced to provide a mechanism for oxidative phosphorylation in mitochondria, also implicates a direct functional link between ATP synthesis and ion transport (Mitchell, 1961). A later version (Mitchell and Moyle, 1965) even indicates that it will eventually explain the valinomycin-induced cation transport, the main concern of our present paper. The salient features of the hypothesis are the coupling of respiration and phosphorylation *via* the translocation of H^+ and/or OH^- across a "coupling" membrane. The earlier version of the hypothesis called for 1 equiv of H^+ translocation for each ATP synthesized, or hydrolyzed *via* ATPase (*i.e.*, $H^+/\sim P = 1$). In the later version the mechanistic basis for this stoichiometry was abandoned in favor of $2H^+/\sim P$ obtained experimentally. Since the H^+ translocation provides the driving force for K^+ translocation on a one for one basis (*cf.* Chappell and Crofts, 1965), the hypothesis is inconsistent with the high $K^+/\sim P$ values reported here by us. Further analysis of the hypothesis by thermodynamic relationships paralleling those used above to compute the efficiency of K^+ transport brings other inconsistencies to light.

The reference conditions for the thermodynamic calculations originally used by Mitchell (1961), and carried through the revised version of the hypothesis (Mitchell and Moyle, 1965), refer to a $\Delta F'$ of -7.0 kcal to form ATP from ADP and P_i at 25°, pH 7.0, $[P_i] = 1$ M, ADP/ATP = 1 (*i.e.*, "centrally poised"), in the presence of excess Mg^{2+} (Atkinson *et al.*, 1959). The same $\Delta F'$ under the same conditions is given by Benzinger *et al.* (1959). The conditions arbitrarily assumed for calculating the energy necessary to form ATP experimentally were the same as in the reference state except for the lowering of the $[P_i]$ to 10 mM which alters the $\Delta F''$ 2.7 kcal to 9.7 kcal.

The experimental conditions employed in Table I have been deliberately chosen as requiring a higher $\Delta F'$ for ATP formation than the assumed conditions of Mitchell. From the data of Benzinger *et al.* (1959) it can be estimated that 1.1 kcal more are required to form ATP at pH 7.8 than at 7.0, although reducing the reaction temperature from 37 to 25° lowers the free energy requirement 0.2 kcal. Omitting the Mg^{2+} (ensured by inclusion of 0.2 mM EDTA to prevent complexing of the extramitochondrial nucleotides) adds a requirement of 1.7 kcal (Burton, 1959). Sufficient Mg^{2+} obviously remains bound to the mitochondria to support oxidative phosphorylation. The $\Delta F'$ term for the $\Delta F''$ of ATP formation under our experimental conditions is therefore 9.6 kcal. Considering the 100 μM ADP equilibrium concentration from Table I, expt 3, as resulting from the phosphate potential attainable exclusively through oxidative phosphorylation, the chemical concentration term of eq 2 for the $\Delta F''$ of ATP formation, $RT \ln [ATP]/[ADP][P_i]$, becomes 6 kcal. The $\Delta F''$ of ATP formation experimentally attainable is thus 9.6 + 6.0, or 15.6 kcal rather than the 9.6 kcal calculated under the conditions assumed by Mitchell.

According to Mitchell (1961), the driving force for ATP formation is an electrochemical activity gradient which can be resolved into two components, a pH gradient and a membrane potential. Since biological membranes have finite breakdown potentials (*cf.* Coster, 1965) and enzyme reactions are inhibited at an unfavorable pH, the electrochemical activity gradient physiologically attainable would have finite limits, indicated by Mitchell and Moyle (1965) as roughly equivalent to a gradient of about 3.5 pH units. The equivalent pH gradient necessary to derive a given free energy change out of the translocation of H^+ is $\Delta F/2.3RT$ or $\Delta F/1.36$. Thus the equivalent pH gradient required to synthesize ATP under the conditions assumed by Mitchell would be 9.6/1.36 or 7.1 pH units, and under the conditions obtainable experimentally, 15.6/1.36 or 11.5 pH units, both substantially beyond the limits considered as physiologically attainable.

In the later version of the hypothesis, Mitchell and Moyle (1965) cite an experiment indicating that the $H^+/\sim P$ ratio is empirically two, which has the effect of dividing the equivalent pH gradient required by two. This would reduce the requirement for the conditions

assumed by Mitchell to 3.5 pH units, within the physiologically attainable range, but the equivalent pH gradient required under the conditions of Table I, expt 3, 5.7 units, remains beyond.

The validity of two for the $H^+/\sim P$ ratio is moreover open to challenge. Not only has no mechanism been provided to support it, but the experiment upon which it is based is equivocal. The presence of 150 mM KCl in the experimental medium (Mitchell and Moyle, 1965) provides a means for the pH decrease observed by the electrode to arise from an energy-dependent H^+ for K^+ exchange (Moore and Pressman, 1964; Christie *et al.*, 1965; Harris *et al.*, 1966). Although the voltage response of the cation electrode to a given $\Delta[K^+]$ at 150 mM K^+ is only 0.001 that of the pH electrode for an equivalent ΔH^+, we have indeed observed a countermovement of K^+ exceeding severalfold the H^+ movement obtained upon addition of small amounts of H_2O_2 to an anaerobic succinate–rotenone system containing 120 mM KCl. Details of these results will be reported elsewhere. The $H^+/\sim P$ ratio moreover could be flexible and a function of the gradient as we have demonstrated for the $K^+/\sim P$ ratio (Figure 5), and it is therefore a tenuous assumption that the same $H^+/\sim P$ ratio obtained under conditions of virtually zero-pH gradient would also be obtained at gradients sufficient to drive ATP synthesis. Accordingly, despite the numerous references to the chemiosmotic hypothesis as a possible mechanism for mitochondrial ion transport (Saris, 1963; Chance *et al.*, 1964; Lynn *et al.*, 1964; Judah *et al.*, 1965a, b; Chappell and Crofts, 1965; Mitchell and Moyle, 1965), it is inconsistent with the high $K^+/\sim P$ ratios we report here, and, within the limitations of thermodynamic analysis, fails to provide sufficient energy for synthesizing ATP under all conditions experimentally supporting oxidative phosphorylation.

The recent report of the pH gradient driven synthesis of ATP by chloroplast fragments has been interpreted as favoring the chemiosmotic hypothesis (Jagendorf and Uribe, 1966). The data in Table IV of their paper indicate that 33 μM ATP can be synthesized from 110 μM ADP (therefore, final [ADP] = 77 μM) and 1.1 mM P_i at pH 8.3 in the presence of excess Mg^{2+}. The chemical concentration term of $\Delta F''$ for ATP formation, RT ln [ATP]/[ADP][P_i], at the end of the reaction would be 3.5 kcal. From Benzinger *et al.* (1959), $\Delta F'$ under these conditions can be estimated as 8.5 kcal, giving a $\Delta F''$ for ATP formation of 12.0 kcal. The energy available from the pH gradient, maximal initially at 4.3 pH units, is equivalent to $4.3 \times 2.3RT$ or 5.8 kcal/mole of H^+, and would have been considerably dissipated by the time equilibrium with the ADP–ATP system had been achieved. It is thus insufficient to synthesize ATP assuming $H^+/\sim P$ ratios of one, or considered somewhat less likely, two. The possibility cannot be ruled out that the chloroplast experiment represents a true harnessing of the pH gradient to form ATP but at $H^+/\sim P$ ratios higher than called for by the chemiosmotic hypothesis and comparable with the $K^+/\sim P$ ratios we report. Evidence for the existence of an energy-coupled H^+ pump, however, does not mean that it necessarily functions as the mediator between ATP synthesis and energy derived from light.

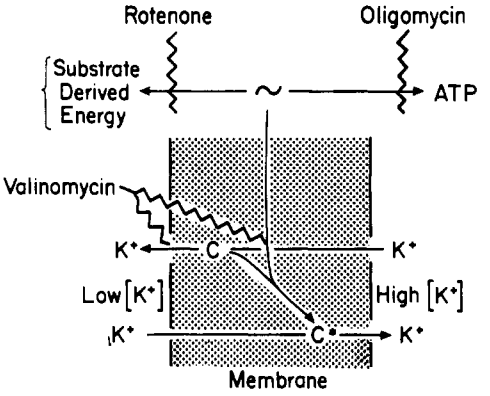

FIGURE 8: Operational scheme for induced mitochondrial ion transport.

A scheme which is compatible with our data is depicted in Figure 8. The energy-transfer pathways operating between the electron transport chain, ATP synthesis, and ion transport are linked through a common component, the energy of which may be denoted as "$\sim$" in keeping with previously proposed mechanisms of oxidative phosphorylation. "$\sim$" could, under appropriate conditions, be transferred to the electron transport chain to effect reversed electron transport, to the reaction sequence leading to ATP synthesis, or to the ion transport carrier C elevating it to the charged state C*. "$\sim$" arises from energy derived from either electron transport or reversa lof the ATP-formation reactions, *i.e.*, ATPase. C* in turn is the energized carrier which actually combines with the cation to be translocated. The formation and utilization of $\sim$ are presumably all or nothing phenomena, as would be consistent with the integral P/O ratios obtained by most investigators, and compatible with the formation and rupture of an energy-rich chemical bond. We presume, however, that C is energized to C* through a molecular conformational change, such as considered earlier by Goldacre (1952), which would permit the binding of variable amounts of cation. Countergradient translocation would occur only when the number of carrier-bound ions did not exceed the limits imposed by a function of the magnitudes of "*" and the gradient. Thus the stoichiometry between the translocated ions and energy equivalents expended, *e.g.*, ATP hydrolyzed, could be nonintegral and flexible to accommodate the variable thermodynamic load imposed by a variable gradient. It would seem reasonable that, in such a model, the discharged carrier would retain some affinity for cations, which accounts for the observed initiation by valinomycin of the facilitated diffusion of K^+ down a preexisting gradient, in the case of limited energy supply and low extramitochondrial [K^+] (*cf.* Figure 5).

The fact that under different conditions valinomycin

promotes either the net influx or net efflux of K^+ can be interpreted as evidence for a primary effect on membrane permeability. Indeed it has been suggested that this is the principal action of the ion transport inducing antibiotics, and that the ion pump is electrogenic in nature, separate and distinct from the membrane pore, which reacts with the inducer to control the specificity and accessibility of cations to the pump (Chappell and Crofts, 1965). Recent work of Mueller and Rudin (1966) indicates that even reconstituted lipid membranes have their permeability to K^+ specifically enhanced by valinomycin. A permeability effect alone, however, cannot explain the ability of valinomycin to reverse the direction of movement of K^+ through the mitochondrial membrane.

We propose that ions traverse mitochondrial membranes through a region which is structurally and mechanistically related to the energy transduction assembly, and thus the action of valinomycin at a membrane site would simultaneously affect both energy transduction capacity and membrane permeability. Such a dualistic action of valinomycin has previously been suggested (Harris *et al.*, 1965, 1966).

The valinomycin-induced transport of K^+ by mitochondria has provided a valuable tool for exploration of the quantitative aspects of ion translocation in biological systems. In this paper we have concentrated on the energetic aspects of the system, and have used the information obtained to set up criteria for the evaluation of previously proposed, as well as newly formulated ion transport mechanisms. The data presently available, in our belief, support only the rather general scheme of transport outlined, but it is hoped that continued study of this system will lead to the formulation of more detailed mechanisms.

Acknowledgments

We wish to acknowledge the valuable technical assistance given by Mr. Graham Catlin and Mr. Louis Olenick.

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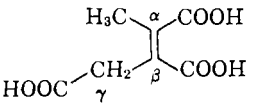
α-Methyl-*cis*-aconitic Acid, *cis*-Aconitase Substrate. I. Synthesis*

Oscar Gawron and Kishan Paul Mahajan†

ABSTRACT: The synthesis of α-methyl-*cis*-aconitic anhydride, a ready source of the new aconitase substrate, α-methyl-*cis*-aconitic acid, is reported herein. Synthesis proceeds *via* condensation of ethyl cyanoacetate with diethyl methyloxalacetate, hydrolysis and decarboxylation of the resulting unsaturated cyano ester to a methylaconitic acid mixture, formation of an intermolecular anhydride from the methylaconitic acids, and partial hydrolysis and isomerization of the dianhydride to give α-methyl-*cis*-aconitic anhydride. The structure of α-methyl-*cis*-aconitic anhydride is deduced from the nmr spectrum and from formation of a complex with hydroquinone. Related compounds and related isomerizations, including a facile thermal isomerization of *trans*-aconitic anhydride to *cis*-aconitic anhydride, are described.

Investigations of substrate structural requirements for *cis*-aconitase (EC 4.2.1.3 aconitate hydratase) having been limited (Dickman, 1961) to geometrical and optical isomers, esters, and fluoro derivatives of the natural substrates, it was deemed of interest to investigate the effect of alteration of the carbon skeleton on substrate activity. As an initial attempt in this direction, the synthesis of α-methyl-*cis*-aconitic acid[1] was undertaken and in this paper and the accompanying paper (Gawron and Mahajan, 1966) the synthesis and substrate properties of the compound are reported. Interest in a methyl substituent was also stimulated by the fact that several α-methyl-substituted dicarboxylic acids are active substrates; L-methylsuccinic acid



α-methyl-*cis*-aconitic acid (I)

for succinic dehydrogenase (Gawron *et al.*, 1962), its oxidation product, methylfumaric acid (mesaconic acid), for L-*threo*-3-methylaspartate ammonia-lyase (EC 4.3.1.2) (Barker *et al.*, 1959), and the amination product, L-*threo*-3-methylaspartic acid, for L-*threo*-3-methylaspartate carboxyaminomethylmutase (EC 5.4.99.1) (Barker *et al.*, 1958).

The synthesis of *cis*-aconitic acid (Malachowski and Maslowski, 1928; Malachowski *et al.*, 1928) proceeds through the synthesis of *cis*-aconitic anhydride, a compound sufficiently stable for unequivocal structure proof and for utility as a source of the relatively unstable *cis*-aconitic acid. By analogy, the synthesis of α-methyl-*cis*-aconitic acid (I) was directed to the synthesis of α-methyl-*cis*-aconitic anhydride (V, Figure 1). Figure 1 gives the sequence of reactions by which a directed synthesis of α-methyl-*cis*-aconitic anhydride was achieved. The brackets about II–IV in Figure 1 are used to indicate that the given structure is but one of several possible structures for the substance in

* From the Department of Chemistry, Duquesne University, Pittsburgh, Pennsylvania. *Received February 25, 1966.* This work was supported by a research grant (GM-06245) from the National Institutes of Health, U. S. Public Health Service. A preliminary report of this work has been presented (Gawron and Mahajan, 1965).

† Abstracted in part from the Ph.D. thesis of K. P. M., Aug 1965.

[1] The nomenclature of Rogerson and Thorpe (1906) for this compound has been revised to correspond with one of the products, α-methylisocitric acid, obtained by *cis*-aconitase-catalyzed hydration. The compound has previously been referred to as γ-methyl-*cis*-aconitic acid (Gawron and Mahajan, 1965).