still represents a minimal, structural framework of the cell membrane, made up of reduced numbers of lipid and protein molecules in comparison to the untreated stroma. It must be recalled, however, that the residual stroma resulting from treatment with hypertonic saline has lost its original stability. Thus, the structural integrity of the cell membrane might be dependent not only upon a minimal lipid–protein framework but also upon the presence of additional proteins and/or lipoproteins to stabilize the minimal layer.

Acknowledgment

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Valinomycin and Mitochondrial Ion Transport*

Etsuro Ogata and Howard Rasmussen

ABSTRACT: The effects of valinomycin upon K^+ and Ca^{2+} transport in the rat liver mitochondria have been studied. The results indicate that valinomycin acts by altering the permeability of the membrane to K^+, and that under these conditions K^+ and Ca^{2+} compete for the same carrier. It is concluded that the mitochondrial swelling associated with K^+ uptake is an osmotic phenomenon.

Although mitochondrial potassium uptake and binding have been studied for a number of years (Stanbury and Mudge, 1953; Gamble, 1957; Amoore, 1960), only recently have agents been discovered which dramatically alter this mitochondrial activity. Moore and Pressman found that the antibiotic, valinomycin, stimulated K^+ uptake and H^+ ejection in an energy-dependent reaction (Moore and Pressman, 1964; Pressman, 1964). This was followed by the demonstration that parathyroid hormone stimulates K^+ uptake under appropriate circumstances (Rasmussen *et al.*, 1964).

Moore and Pressman (1964) have proposed that valinomycin acts to promote K^+ uptake by increasing

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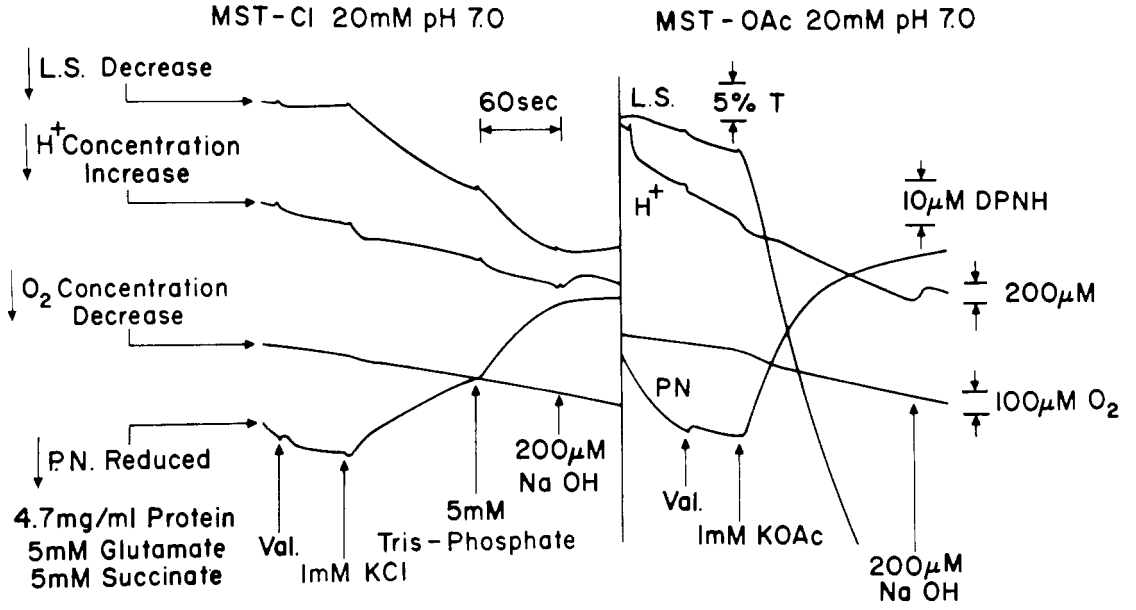

FIGURE 1: The responses of mitochondria (4.7 mg of protein/ml) to K^+ addition in a medium (mannitol 120 mM, sucrose 50 mM, Tris–succinate 5 mM, Tris–glutamate 5 mM, pH 7.0) with either 20 mM Tris–chloride (MSTCl) or 20 mM Tris–acetate (MSTOAc). From top to bottom are recorded light scattering (540 mμ), H^+ concentration, oxygen concentration and pyridine nucleotide fluorescence (366 → 450 mμ). They were measured simultaneously. Additions were valinomycin 0.06 μg/ml, KCl 1 mM, KOAc 1 mM, Tris–phosphate 5 mM, and NaOH 200 μM. The arrows on the left represent, respectively, from top to bottom, a decrease in light scattering at 540 mμ (L.S.), an increase in hydrogen ion concentration (H^+ ejection), a decrease in oxygen concentration, and an increased reduction of pyridine nucleotides. The values between lines bracketed with arrows on the right are the calibrations of the respective measurements.

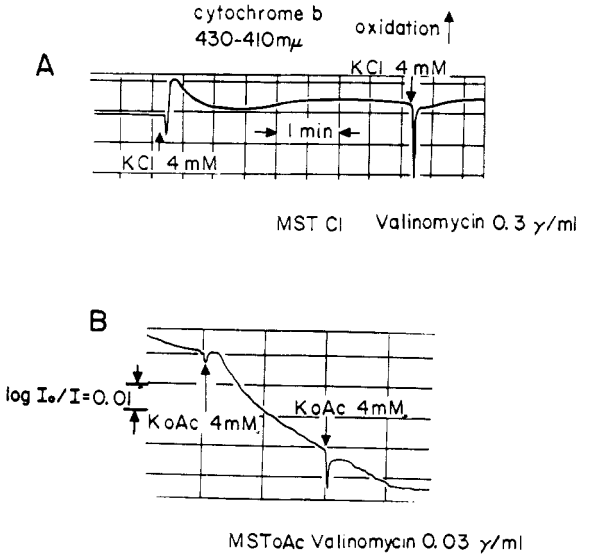

FIGURE 2: The response of cytochrome *b* (430–410 mμ) of the valinomycin pretreated mitochondria (3.6 mg of protein/ml) to the addition of K^+ in a MSTCl medium (A) and a MSTOAc medium (B). Incubations as in Figure 1. Reactions proceeded from left to right and two vertical divisions correspond to 1 min.

the synthesis of a phosphoprotein specifically involved in ion transport. If this is the case, it would be difficult to account for the high degree of cation specificity involved in this reaction without making the further assumption that either a variety of cation-specific phosphoproteins exist or that the transport of the other cations, calcium, magnesium, and manganese, takes place by a fundamentally different mechanism. One or the other of these assumptions is necessary to account for the fact that valinomycin has no apparent stimulating effect upon the transport of other cations which are translocated across the mitochondrial membrane.

One of the major reasons for the Moore and Pressman proposal was their observation that phosphate was required in order for K^+ and valinomycin to stimulate respiration. Their studies were carried out in chloride media. Recent studies of calcium and potassium uptake in other media indicate that the mitochondrial membrane is relatively impermeable to chloride as well as to potassium (Rasmussen *et al.*, 1964, 1965; Judah *et al.*, 1965). However, acetate is a permeant anion in the presence of which K^+ and Ca^{2+} accumulation can occur (Rasmussen *et al.*, 1964, 1965).

The initial purpose of the present study was that of examining the effect of acetate upon the response of mitochondria to valinomycin and potassium. The re-

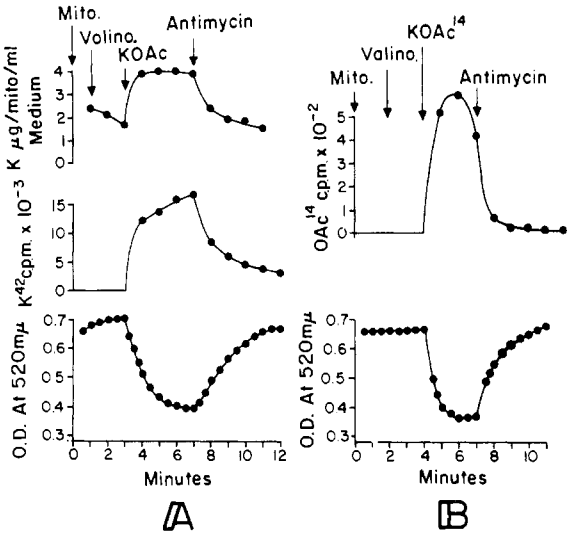

FIGURE 3: The correlation between mitochondrial swelling and ion accumulation in the presence of valinomycin. In a test tube fitted in a Coleman photometer, 40 ml of the medium (sucrose 250 mM, Tris–succinate 6 mM, Tris–glutamate 6 mM, $MgCl_2$ 1 mM, mitochondria of 0.5 mg of protein/ml, pH 7.4) was added with valinomycin (0.6 μg/ml in final) followed by either ^{42}K potassium acetate (4 mM) (A) or ^{14}C potassium acetate (4 mM) (B) and then by antimycin to a final concentration of 1 μg/ml. Aliquots (3 ml) were withdrawn at the times indicated in the figure and washed onto the Celite pad. The radioactivity on the pad was counted after the pad had been washed with cold 0.25 M sucrose and dried. Total potassium in a perchloric acid extract of the pad was measured by flame photometry. The intensity of transmitted light was adjusted to an optical density reading of 0.5–0.7 at time zero and the readings on this scale were recorded in relative rather than absolute units. A decrease in the reading corresponds to mitochondrial swelling.

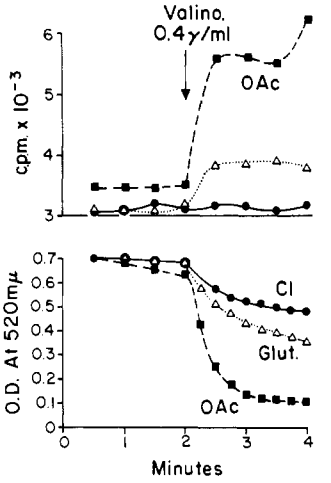

FIGURE 4: The influence of anions upon mitochondrial swelling and ion uptake in the presence of valinomycin. Valinomycin was added to 10 ml of medium which consisted of mannitol 200 mM, sucrose 50 mM, potassium glutamate 5 mM, Tris salts of either ^{14}C-acetate, ^{36}Cl-chloride, or ^{14}C-glutamate 50 mM, pH 7.4, and mitochondria of 4.3 mg of protein/ml. Care was taken to obtain nearly the same amount of radioactivity in each medium (about 5×10^5 cpm/ml). Optical density readings and radioactivity in the isolated mitochondria are plotted as a function of time as in Figure 3. ●——● chloride medium; Δ- - -Δ, glutamate medium; ■- - -■, acetate medium.

sults of these studies led naturally to a reconsideration of the mechanism of action of valinomycin and eventually to an examination of the possible relationship between calcium and potassium transport.

Methods

Rat liver mitochondria were prepared by a modification (Rasmussen *et al.*, 1965) of the method of Schneider (1958) and finally suspended in 0.37 M sucrose. Accumulation of chloride, acetate, and glutamate was measured by counting ^{36}Cl and ^{14}C isotopes trapped on Celite planchets (Azzone and Ernster, 1961). Potassium binding was measured by one of the several methods according to the purposes of the experiments: (1) Concentration of potassium was measured with a potassium electrode (Beckman cationic electrode 39137) connected to a Beckman Expandomatic pH meter and a Varian recorder. This method was restricted to cases where the interference from the other cations was minimal. (2) Gross uptake was measured by counting ^{42}K trapped on the Celite planchets. (3) After extraction of the Celite pad with perchloric acid, net amount of potassium was measured flame photometrically in a Zeiss spectrophotometer (Rasmussen *et al.*, 1964). (4) Release of endogenous potassium was followed by counting ^{42}K remaining on the Celite planchets. In this case, mitochondria were labeled with ^{42}K by a 10-min incubation at 38° in a medium that consisted of bovine serum albumin 0.1%, Tris adenosine triphosphate (ATP)[1] 1 mM, magnesium chloride 1 mM, sodium succinate 10 mM, Tris–chloride 10 mM (pH 7.4), ^{42}K potassium chloride 40 mM, and sucrose 210 mM. The labeled mitochondria were washed once with the medium of the experiment before use. Calcium uptake was measured by the millipore technique (Rasmussen *et al.*, 1965). Oxygen consumption was measured with a vibrating platinum electrode. Oxidation and reduction of cytochrome *b* was measured by direct spectrophotometric method in an Aminco-Chance dual wavelength spectrophotometer using a wavelength pair of 430 and 410 mμ (Chance and Williams, 1956; Chance

[1] Abbreviations: ATP, adenosine triphosphate; MSTCl, mannitol sucrose Tris–chloride medium; MSTOAc, mannitol sucrose Tris–acetate medium; TMPD, tetramethyl-*p*-phenylenediamine.

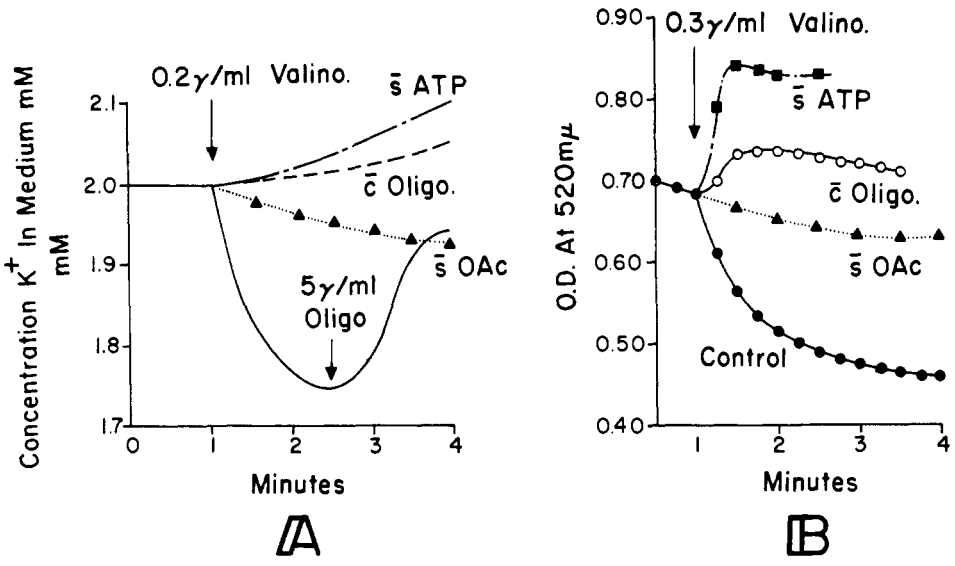

FIGURE 5: The changes in mitochondrial volume and K^+ concentration in the medium induced by valinomycin when ion uptake was supported by ATP. (A) Potassium concentration in the medium: mitochondria (3.2 mg of protein/ml) were incubated for 1 min in a medium of Tris ATP 3 mM, antimycin 2 μg/ml, Tris-acetate 50 mM, mannitol 120 mM, sucrose 50 mM, Tris-glutamate 5 mM, Tris-succinate 5 mM, and potassium glutamate 2 mM, pH 7.4. Valinomycin was then added and the change of potassium concentration in the medium was recorded with a cation electrode. ———, control; oligomycin 5 μg/ml was added at the time indicated by the arrow. ▲– – –▲, without acetate; Tris-acetate was replaced by 80 mmoles of mannitol. – – – –, with oligomycin; oligomycin 5 μg/ml was added with the valinomycin. — · —, without ATP; Tris ATP was omitted. (B) Swelling: optical density was recorded as in Figure 3 before and after the addition of valinomycin to the mitochondria (2.0 mg of protein/ml) incubated in a medium containing Tris ATP 3 mM, antimycin 2 μg/ml, Tris-acetate 50 mM, potassium acetate 5 mM, mannitol 120 mM, and sucrose 50 mM, pH 7.4. ●——●, control. ▲– – –▲, without acetate; Tris acetate was replaced by 80 mmoles of sucrose. O– – –O, with oligomycin; oligomycin 5 μg/ml was added with the valinomycin. ■— —■, without ATP; Tris ATP was omitted.

and Yoshioka, 1965). The swelling experiments were conducted by measuring the absorbance at 520 mμ in a Coleman Junior spectrophotometer. The light intensity was adjusted by galvanometer controls to produce a reading of 0.5–0.7 on the absorbance scale at zero time. The changes recorded were in relative rather than absolute units. The increase and decrease of the intensity recorded by this method corresponded well with morphological swelling and contraction as verified by electron microscopic examination of suitably fixed preparations of mitochondria (H. Rasmussen, H. Ris, and E. Ogata, in preparation). pH was recorded with the Varian recorder and Beckman Expandomatic pH meter. Pyridine nucleotides fluorescence was measured by exciting with light of 366 mμ and recording the resulting fluorescent emission at 450 mμ. In other experiments oxygen consumption (vibrating electrode), pyridine nucleotides fluorescence (366 → 450 mμ), pH, light scattering (at 540 mμ), and potassium concentration (potassium electrode) were measured simultaneously employing an apparatus constructed by the electronics instrument shops of the Johnson Foundation, University of Pennsylvania.[2] Incubation was carried out at room temperature (20–24°). The exact constituents of the medium are recorded in the legend of each figure. Valinomycin,[3] antimycin, and oligomycin were dissolved in 95% ethanol in concentrated forms so that an addition of 10 μl of the particular solution to 5–10 ml of media gave the proper final concentration. Mitochondrial protein concentration was measured using the biuret method (Gornall *et al.*, 1949). All the results reported were highly reproducible, and each figure represents a typical example of the responses observed under the particular condition.

Results

Valinomycin Effects in Chloride and Acetate Media. A comparison of the responses of mitochondria to potassium addition, in the presence of valinomycin, in an acetate as compared to chloride medium is shown in Figure 1. The responses in the two environments were quite different. In a chloride medium, K^+ addition was followed by prompt H^+ ejection (160 μM), a barely

[2] We are indebted to Dr. Britton Chance and Mr. Dieter Meyer for the design and construction of this apparatus.

[3] The valinomycin was a gift from Dr. Henry A. Lardy.

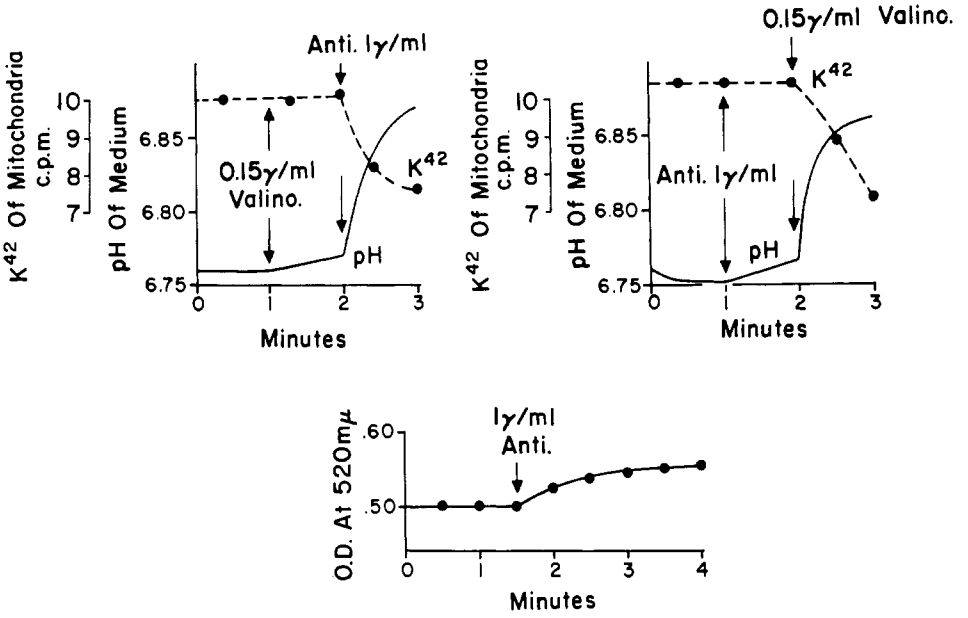

FIGURE 6: Potassium and hydrogen exchange induced by valinomycin in the absence of energy source. Figures above: valinomycin and antimycin were added to mitochondria which had accumulated ^{42}K at times as indicated by the arrows. The reaction medium consisted of sucrose 240 mM, $MgCl_2$ 0.06 mM, sodium glutamate 12.5 mM, and mitochondria (1 mg of protein/ml). The pH and release of ^{42}K from the mitochondria were followed as a function of time. The ^{42}K-tagged mitochondria were obtained by previously incubating the mitochondria with a medium containing ^{42}K, ATP, and succinate. Figure below: Antimycin was added to mitochondria pretreated with valinomycin and the change of optical density was followed as a function of time. The medium contained valinomycin 0.15 μg/ml, mannitol 200 mM, sucrose 20 mM, Tris–glutamate 5 mM, and mitochondria (1.5 mg of protein/ml). Note that mitochondrial contraction occurred when K^+ ejection took place.

perceptible increase in rate of oxygen consumption, a moderate decrease in light scattering (swelling), and a slow oxidation of the pyridine nucleotides. The subsequent addition of phosphate led to enhanced swelling, respiration, and pyridine nucleotides oxidation. The addition of K^+ in the acetate medium led to little H^+ ejection, a definite and sustained increase in respiration, a prompt and profound decrease in light scattering, and a rapid oxidation of the pyridine nucleotides. The subsequent addition of phosphate (not shown in Figure 1) did not lead to any further changes. The addition of similar concentrations of potassium to mitochondria not treated with valinomycin had very little effect upon any of the parameters studied except that potassium acetate addition did induce a very slight decrease of light scattering (mitochondrial swelling).

The responses of other respiratory carriers to K^+ addition were also altered by valinomycin. In the absence of the antibiotic, K^+ addition had little effect upon the absorbance difference at 430–410 mμ. In the presence of valinomycin, K^+ addition led to a cyclic oxidation–reduction of cytochrome *b* when the mitochondria were incubated in a chloride medium (Figure 2). A second addition of K^+ led to no further response (Figure 2), a situation analogous to that noted upon the successive additions of calcium in a chloride medium, in the absence of valinomycin (Rasmussen *et al.*, 1965). In the acetate medium, there was a progressive "apparent" reduction after a very slight initial oxidation. This may be due to the marked swelling (Figure 1) which occurs in this circumstance and not a true reduction of the cytochrome.

A study of the relationship between the swelling observed in an acetate medium (Figure 1) and the uptake of both acetate and potassium revealed that both ions were accumulated (Figure 3), and the extent of swelling was proportional to ion accumulation. Furthermore, if antimycin A, an inhibitor of electron transport, was added to mitochondria which had accumulated potassium acetate, both ions were ejected with a simultaneous increase in light absorption (mitochondrial contraction) as shown in Figure 3. The time courses of ion ejection and mitochondrial contraction were nearly identical. In the presence of antimycin A, the addition of tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate reestablish electron transport through the terminal portion of the electron transport chain (Jacobs, 1960). The addition of TMPD and ascorbate, under the present conditions, led to a reaccumulation of K^+ and acetate and a return of the mitochondria to the swollen state.

The results with acetate led to an investigation of the basis of the swelling observed in the chloride medium (Figure 1). This was considerably less than that ob-

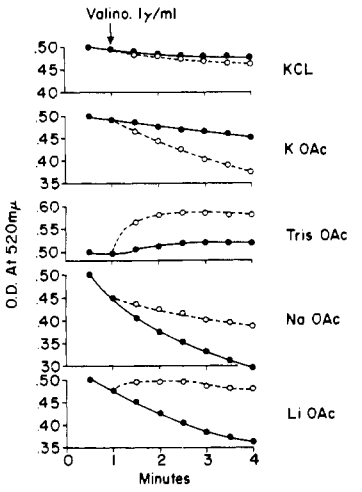

FIGURE 7: The effect of valinomycin upon mitochondrial swelling and shrinkage in the absence of energy source. Mitochondria were added at zero time to the final concentration of 3.8 mg/ml. One minute later, valinomycin was added and optical density at 520 mμ followed as a function of time. Arbitrary units were used to record optical density as in Figure 3. The medium contained sucrose 40 mM, oligolycin 7 μg/ml, antimycin 1 μg/ml, and Tris–chloride or Tris–acetate 10 mM, pH 7.4. In addition the media contained 200 mM of their respective salts, KCl, KOAc, NaOAc, and LiOAc. The medium labeled Tris OAc contained 10 mM Tris–acetate and 400 mM mannitol in addition to the basic constituents. ●——●, control; O– – –O, valinomycin 0.3 μg/ml.

served when acetate was the major anion. Nonetheless, it was not possible to demonstrate any uptake of labeled chloride (^{36}Cl) (Figure 4), an observation in keeping with much indirect evidence that the mitochondrial membranes are impermeable to chloride. When glutamate or succinate replaced chloride as the major anion, significant swelling and accumulation of the labeled anion (^{14}C) was observed (Figure 4). Thus, the swelling observed in the chloride medium may be due to the accumulation of substrate plus the greater dissociation of KA as compared to HA, where A^- represents unknown anionic sites within the mitochondria. The addition of permeant anions such as acetate or phosphate, to mitochondria pretreated with valinomycin and K^+, led to a prompt increase in K^+ accumulation and mitochondrial swelling.

The accumulation of K^+, in the presence of valinomycin, can be supported by ATP hydrolysis rather than substrate oxidation (Moore and Pressman, 1964). The effect of an acetate environment upon ATP-supported ion accumulation and mitochondrial swelling was studied. The results are shown in Figure 5. There was a reasonably good correlation between swelling and the disappearance of K^+ from the medium (as measured with a K^+ electrode). Both were prevented or reversed by the addition of oligomycin B. This result contrasts with that obtained with the parathyroid hormone (Rasmussen *et al.*, 1964), in which case ATP-supported K^+ uptake could not be demonstrated even though substrate-supported uptake was readily demonstrable.

Valinomycin Action during Inhibition of Energy Supply. Valinomycin was capable of influencing K^+ and H^+ movements even under circumstances where energy supply was blocked. In a K^+-free medium the addition of either valinomycin or antimycin singly had no effect upon K^+ release or H^+ uptake. However, the successive addition of both agents led to a release of K^+, an uptake of H^+, and mitochondrial contraction (Figure 6). The results recorded here were obtained by measuring ^{42}K ejection from mitochondria. Similar results were obtained with a potassium electrode. The H^+ uptake was pH dependent. Appreciable uptake of H^+ did not occur when pH was 7.8 or greater, although K^+ release was considerable even at pH 8.0.

If similar experiments were carried out in medium containing high concentrations of K^+, antimycin alone had no effect upon swelling but valinomycin added subsequently promoted swelling (Figure 7). The swelling was again anion dependent suggesting an accumulation of both K^+ and permeant anions. When Tris, sodium, or lithium replaced K^+, inhibition of swelling or contraction rather than swelling occurred. The swelling promoted by valinomycin in the antimycin-inhibited system was not blocked by oligomycin, ruling out the possibility that endogenous ATP was supporting this uptake.

Competition between Ca^{2+} and K^+ Uptake. The results recorded in Figures 1 and 2 indicate that some of the responses of mitochondria to K^+, in the presence of valinomycin, are similar to those observed upon Ca^{2+} addition, suggesting that K^+ and Ca^{2+} might be transported by similar mechanisms. For this reason it became of interest to examine the effect of valinomycin upon the response of mitochondria to Ca^{2+} in the presence and absence of K^+. Valinomycin had several important effects in the absence of added K^+. As shown in Figure 8, Ca^{2+} addition in a chloride medium led to the expected H^+ ejection, burst of respiration, and oxidation–reduction cycle of pyridine nucleotides. There was no K^+ ejection. The addition of Ca^{2+} to valinomycin-treated mitochondria led to the same stimulation of respiration and reduction of pyridine nucleotides, but H^+ ejection was less and significant K^+ ejection occurred accompanied by an increase in light scattering (contraction). In the absence of valinomycin, 1.11 μmoles of H^+ was ejected/μmole of Ca^{2+}, whereas in the presence of valinomycin only 0.84 μmole of H^+ and 0.42 μmole of K^+ were ejected. Similarly in an acetate medium (Figure 9), Ca^{2+} addition led in both cases to a typical oxidation–reduction cycle of pyridine nucleotides, a burst of respiration, but in the presence of valinomycin K^+ ejection (32 μM) was observed, less H^+ was ejected (70 μM compared to 90 μM), and there was less mitochondrial swelling. The results seemed to indicate that the rate of the reaction of Ca^{2+} with mitochondria was not altered by valinomycin addition, at least as measured by the time for the completion of the

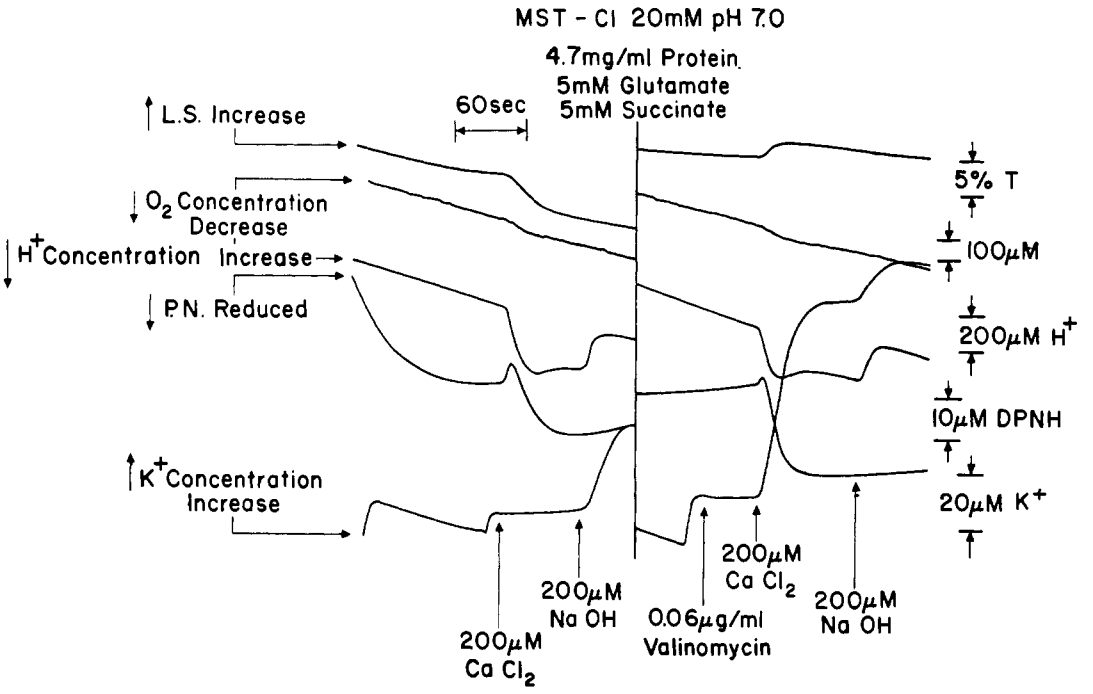

FIGURE 8: The effect of valinomycin upon the responses of mitochondria (4.7 mg of protein/ml) to calcium addition in MSTCl medium. From top to bottom are recorded light scattering (540 mμ), oxygen concentration, H^+ concentration, pyridine nucleotides fluorescence, and K^+ concentration. See Figure 1 for explanation of notations. Note particularly that when valinomycin was present (right side) the addition of calcium led to significant K^+ ejection and a less marked H^+ ejection than did a similar addition of calcium in the absence of valinomycin (left).

cyclic oxidation–reduction of pyridine nucleotides in an acetate medium. However, as shown in Figure 10, when the behavior of cytochrome *b* in a chloride medium was observed, valinomycin appeared to increase the rate of the cyclic oxidation–reduction of cytochrome *b* induced by calcium (compare Figure 10B with 10A), and valinomycin plus K^+ decreased the rate considerably (Figure 10C).

The competition between calcium and potassium accumulation, in the presence of valinomycin, was studied further by examining the effects of each ion upon the uptake and retention of the other. The results are shown in Figure 11. When both ions were added simultaneously (Figure 11A) Ca^{2+} was accumulated first. K^+ accumulation commenced only after much of the calcium had disappeared from the medium. The addition of calcium after K^+ accumulation had begun led to prompt ejection of K^+ (Figure 11B) which was reaccumulated after the calcium had been accumulated. When the order of addition was reversed, K^+ addition had little effect upon the rate or extent of calcium accumulation. However, if the K^+ concentration was increased to 10 mM (rather than 1 mM), prompt Ca^{2+} ejection was observed (Figure 11C). None of these effects of K^+ were observed if valinomycin was absent.

A most interesting effect of valinomycin upon the rate of antimycin A induced release of accumulated calcium is recorded in Figure 12. Calcium taken up during substrate oxidation was released by the addition of antimycin A. The rate of this release was less rapid if valinomycin were present, a condition under which the release of endogenous K^+ is taking place (Figure 6). This effect of valinomycin was reversed by the addition of K^+ to the medium, a condition known to inhibit the release of endogenous K^+.

Discussion

The present results confirm and extend those of Moore and Pressman (1964) and of Pressman (1965). They indicate that valinomycin has a profound and significant effect upon the energy-dependent uptake of potassium by isolated liver mitochondria, that this effect is related to a change in the K^+ permeability of the mitochondrial membrane, and that K^+ transport, induced by valinomycin, and Ca^{2+} transport have a number of similarities. Our results do not support the original proposal of Moore and Pressman (1964), that the action of this antibiotic is upon the synthesis of a specific phosphoprotein. Furthermore, we have found, in contradistinction to Moore and Pressman, that phosphate is not specifically required in order for K^+ and valinomycin to stimulate respiration. Potassium and valinomycin can stimulate respiration in the absence of added phosphate as long as a permeant anion is present. This increased respiration is the result of an increased rate of K^+ influx (Figures 1–3). Our data go further and indicate that a significant effect of valino-

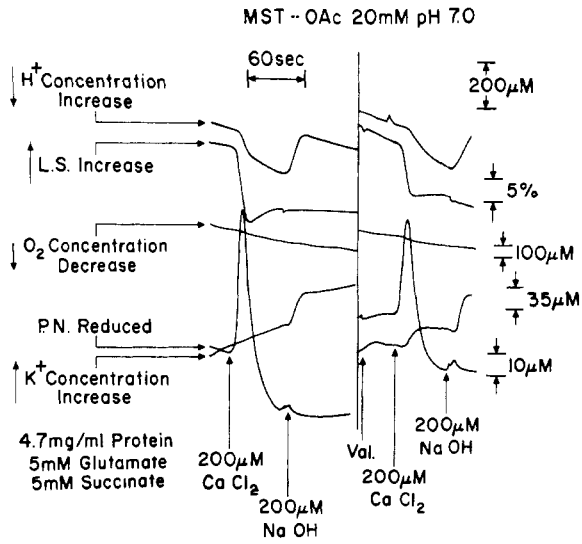

FIGURE 9: The effect of valinomycin (0.06 μg/ml) upon the responses of mitochondria (4.7 mg of protein/ml) to calcium addition in MSTOAc medium. From top to bottom are recorded H^+ concentration, light scattering (540 mμ), oxygen concentration, pyridine nucleotides fluorescence, and K^+ concentration in the medium. An upward deflection of the trace of pyridine nucleotides fluorescence corresponds to an oxidation of pyridine nucleotides.

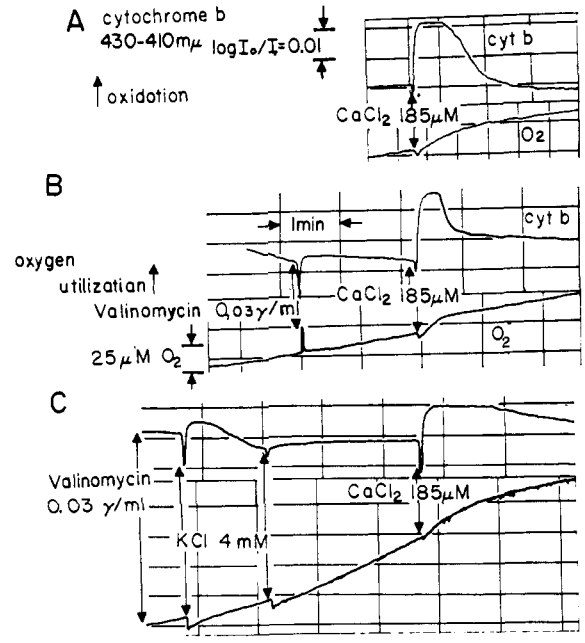

FIGURE 10: The effect of valinomycin and potassium upon the responses of mitochondria (3.6 mg of protein/ml) to calcium in MSTCl medium. The changes in cytochrome *b* absorbance and oxygen concentration are plotted in each figure. (A) The response in the absence of both valinomycin and K^+. (B) The response in the presence of valinomycin. (C) The response in the presence of both valinomycin and K^+.

mycin upon K^+ translocation can be observed in the absence of both ATP hydrolysis and electron transport (Figures 6 and 7). These data constitute good evidence for the hypothesis that valinomycin either specifically alters the permeability of the mitochondrial membrane to K^+, or that it increases the affinity of cation carrier(s) for K^+. The first of these two alternatives is similar to the mechanism of gramicidin action proposed by Chappell and Crofts (1965). The effect this change will have upon K^+ movement depends upon the metabolic state of this membrane, the composition of the external medium, and the intramitochondrial content of ions. Furthermore, the present evidence indicates that H^+ can move inward, in exchange for K^+, across the mitochondrial membrane independently of the energy supply (Figure 6), constituting another argument against the H^+ pump model of ion transport (Mitchell, 1961) across this membrane. This may be explained by considering that the membrane itself is relatively permeable to H^+ which can move passively along the electrochemical potential produced by the translocation of the other ions.

An additional argument against the hydrogen pump hypothesis is the present observation (Figures 8 and 9) that calcium addition in the presence of valinomycin leads to K^+ ejection as well as H^+ ejection. The total concentration of monovalent cation released under these circumstances is nearly identical with the amount of H^+ released when no valinomycin is present. Thus in the presence of valinomycin, K^+ can partially replace H^+ ejection, a phenomenon not easily accounted for by the H^+ pump hypothesis.

The present data concerning the interactions between K^+ and Ca^{2+} uptake in the presence of valinomycin (Figures 8–12) lead to the conclusion that some type of competition exists between the transport of these two ions. They certainly compete for the same energy source and the data are compatible with the view that they are transported by the same carrier. In particular, the results shown in Figure 12 indicate that the passive fluxes of these two ions may be competitive because the addition of valinomycin with a consequent increase in passive K^+ flux (Figure 6) led to a decrease in the rate of calcium release, suggesting a competition between K^+ and Ca^{2+} for the same carrier involved in the facilitated release of these ions from the mitochondria.

The alterations in mitochondrial volume observed under the present conditions can all be explained in terms of changes in intramitochondrial osmotic activity. It is particularly significant that swelling and contraction were observed under circumstances where energy supply was blocked. These results are in keeping with previous observations (Rasmussen *et al.*, 1964, 1965; Chappell *et al.*, 1965) concerning the relationship between changes in intramitochondrial ionic composition and mitochondrial volume. They all imply that this

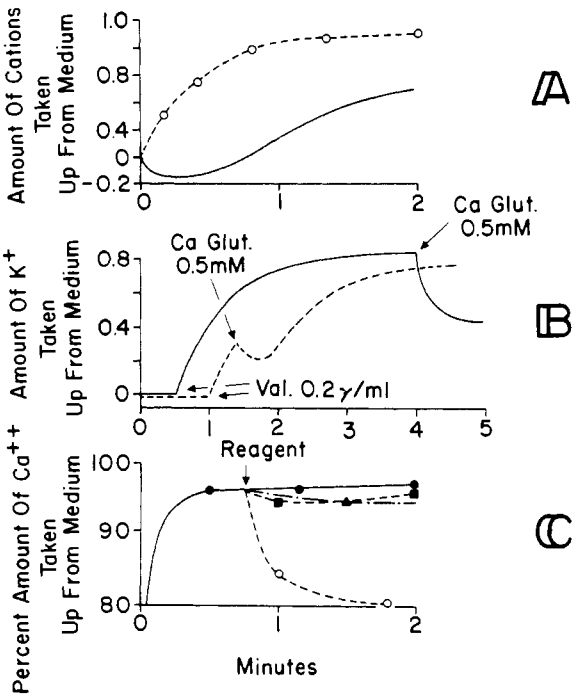

FIGURE 11: The mutual interactions between calcium and potassium uptake by mitochondria in the presence of valinomycin. (A): The case where both cations are available at the same time. At zero time ^{45}Ca-calcium glutamate (0.9 mM), potassium glutamate (1.0 mM), and valinomycin (0.3 μg/ml) were added to mitochondria (3.9 mg of protein/ml) in MSTOAc medium. The concentration of potassium ion was measured by a potassium electrode, and that of calcium by the millipore filter technique. The amount taken up is expressed as millimolar decrease of the respective ions from the medium. ———, potassium; O– – –O, calcium. (B): Calcium added after the uptake of potassium had begun. Valinomycin and calcium glutamate were added, at the time indicated by the arrows, to mitochondria (3.9 mg of protein/ml) in MSTOAc medium containing 1 mM potassium glutamate. Concentration of potassium was measured by the potassium electrode method. ———, calcium was added after the maximal uptake of potassium had taken place; – – – –, calcium was added before the maximal uptake of potassium had taken place. (C): Potassium added after the uptake of calcium had been completed. The experiment was initiated by incubating mitochondria (2.9 mg of protein/ml) in MSTOAc medium containing 0.35 mM ^{45}Ca-calcium glutamate. Forty-five seconds later the appropriate reagents were added. The calcium concentration in the medium is plotted as a function of time. ●——●, Tris-glutamate 5 mM; ▲— · —▲, valinomycin 0.3 μg/ml; ■– – –■, potassium glutamate 5 mM; O– – –O, valinomycin 0.3 μg/ml, and potassium glutamate 5 mM.

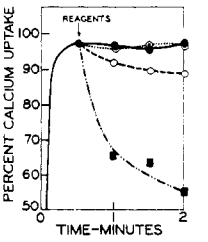

FIGURE 12: The effect of valinomycin and potassium upon the antimycin-induced release of accumulated calcium. At time zero, mitochondria (4.6 mg of protein/ml) were added to MSTOAc medium containing 0.45 mM ^{45}Ca-calcium glutamate. Aliquots were withdrawn at 30-sec intervals and the radiocalcium content of the medium measured. After 30 sec of incubation addition of one or more reagents was made. The percentage of the total calcium which was accumulated by the mitochondria is plotted as a function of time. ●——●, no addition; O– – –O, antimycin 4 μg/ml; ⊙– – –⊙, antimycin 4 μg/ml and valinomycin 0.6 μg/ml; ■— · —■, antimycin 4 μg/ml, valinomycin 0.6 μg/ml, and potassium acetate 20 mM.

type of swelling and contraction is based upon alterations in the osmotic forces across the mitochondrial membranes and not upon the contractile properties of the membrane (Lehninger, 1962). It is of interest that Dilley (1964) has observed a light-induced K^+ efflux and an associated contraction of chloroplasts. His results agree well with our observation of Ca^{2+}-induced K^+ ejection and mitochondrial contraction (Figures 8 and 9) and could be explained by assuming that fixed anionic charges (A^-) within the mitochondria bind K^+ less avidly than either H^+ or Ca^{2+}, *e.g.*, the dissociation constant of KA > HA > CaA. This order is based upon the present observations, those of Dilley, and the fact that Ca^{2+} addition in a chloride medium characteristically leads initially to contraction of mitochondria (Chance, 1965). The view that mitochondrial swelling associated with ion translocations is a consequence of changes in osmotic forces has been challenged by Pressman (1965). He has calculated that the swelling induced by valinomycin in a chloride medium containing 15 mmoles of KCl is some 4–5 times greater than that predicted from the amount of K^+ taken up. This calculation is based upon several assumptions which may not be valid. Pressman has offered as an alternate proposal the thesis that ion flux and a mechano-enzyme system are closely linked phenomena apparently sharing a common intermediate. A somewhat similar proposal has been made by Connelly and Lardy (1964), who propose that the extent of swelling is controlled by the concentration of a high-energy intermediate linked to the oxidative phosphorylation system. However, neither of these views is compatible with the observations that both swelling and contraction can be produced by valinomycin in the absence of energy supply, and that in the case of swelling induced by Ca^{2+} contraction can be brought about either by dicoumarol, antimycin (accompanied by ejection of Ca^{2+}), or inorganic phosphate even under anaerobic conditions (Chance, 1965; Rasmussen *et al.*, 1965). In each of the first two

cases of Ca^{2+}-induced swelling, the rate and extent of mitochondrial contraction correlates well with the rate and extent of Ca^{2+} ejection. In the third case, phosphate uptake leads to acetate ejection and the precipitation of the calcium phosphate within the mitochondria. Likewise K^+ which has accumulated in the presence of valinomycin is lost upon the addition of either dicoumarol or antimycin accompanied by the contraction of the mitochondria. It is not yet possible to state that all mitochondrial swellings and contractions are osmotically induced, but a strong case can be made for those instances where ion accumulation correlates well with a change in volume.

The present results with valinomycin are similar in some respects to those reported with parathyroid hormone (Rasmussen *et al.*, 1964), but there are important differences between the effects of the two agents. All of the effects observed with valinomycin are dependent either upon the addition of external K^+ or are linked to K^+ efflux from the mitochondria, whereas hormone influences Mg^{2+} as well as K^+ transport. Also hormone does not influence ATP-supported K^+ accumulation, as does valinomycin (Figure 5). It is clear that the two agents interact with mitochondria in a significantly different manner even though they share the common ability of being able to promote energy-linked K^+ accumulation when substrate oxidation is the energy source.

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