MECHANISM OF ACTION OF VALINOMYCIN ON MITOCHONDRIA

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Valinomycin has been reported to uncouple oxidative phosphorylation (McMurray and Begg, 1959), although its action on mitochondria differs significantly from that of other uncouplers such as dinitrophenol (DNP) (Pressman, 1963 a). The characteristic energy dependent ejection of H^+ from mitochondria catalysed by this antibiotic was observed to parallel that which accompanies the mitochondrial uptake of divalent ions (Brierley, 1963 Chappell et al, 1963). Evidence will now be presented that the mode of action of valinomycin is indeed mediated via an ion transport phenomenon, namely the energy dependent accumulation of K^+.

The uncoupling activity of valinomycin was measured with the oxygen polarograph (Hagihara, 1961) in terms of the release of respiratory control of an acceptor-free rat liver mitochondrial system. In Fig. 1, Exp. A, stimulation of respiration by valinomycin is totally dependent on K^+ and increases with ascending K^+ concentration. Rb^+ or Cs^+ will substitute for K^+, Na^+ and Li^+ will not. The apparent Km for K^+, Rb^+ and Cs^+ are identical, 5 mM.

The valinomycin stimulated respiration is also totally dependent on Pi (Exp. B). Arsenate can replace Pi. Precise estimation of the Km for Pi is hindere by a marked respiratory response lag at lower concentrations, but its upper limit of 20 μM is considerably lower than the 130 μM Km found by Chance and Hagihara (1963) for oxidative phosphorylation. Accordingly it seems unlikely that Pi exerts its influence via oxidative phosphorylation.

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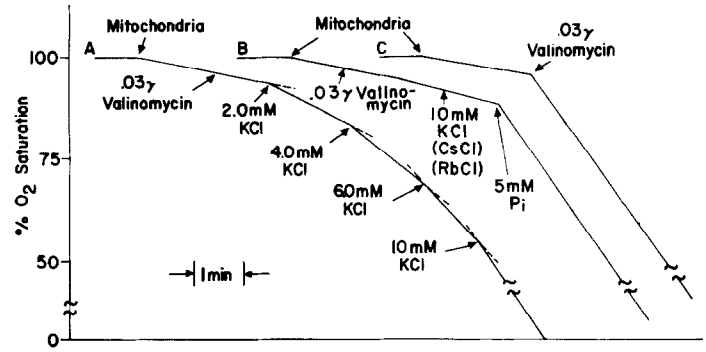

Fig. 1. Release of Respiratory Control by Valinomycin. Basic medium: 0.25 M sucrose; 8.0 mM Tris · HCl, pH 7.4; 3.0 mM succinate; 5.0 mM $MgCl_2$. Total volume in each experiment, 3.5 ml containing 2.5 mg rat liver mitochondrial equivalent protein per ml. Oxygen consumption followed polarographically, T = 22°.

Exp. A. Basic medium plus 5.0 mM. $Na\text{-}PO_4$, pH 7.4. Final KCl concentrations are indicated following successive KCl additions. Exp. B. Basic medium alone, plus additions as indicated. Exp. C. Basic medium plus 5.0 mM Pi and 10 mM KCl.

The inability of K^+ transport to stimulate the respiration of an acceptor free system in the absence of Pi indicates that the energy requirement for saturating mitochondria with K^+ via active transport is quite small. Addition of low concentrations of Pi possibly facilitates the passive diffusion of K^+ from the mitochondria, providing space for the active transport of additional K^+. In this manner a K^+ flux would arise capable of dissipating large quantities of substrate derived energy. Thus the presence of both K^+ and Pi are required for valinomycin to trigger the immediate release of respiratory control typical of other uncoupling agents (Exp. C).

The typical course of ejection of acid from mitochondria initiated by valinomycin is shown in Fig. 2. Like the respiratory control release, the acid production is absolutely dependent on the presence of K^+, Rb^+, or Cs^+ and displays a similar Km toward all three ions, 5 mM. Uncoupling with DNP, or depriving the system from substrate derived energy with antimycin, causes an immediate return to the initial pH. Energy thus appears to be essential for maintaining the valinomycin induced H^+ gradient. H^+ migration can be initiated by adding either K^+ (left tracing) or valinomycin (right tracing) to an otherwise complete system. The subsequent spontaneous reversal of the H^+ gradient at high K^+ concentrations

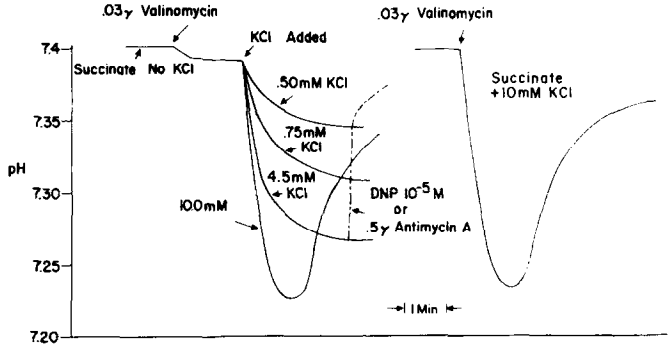

Fig. 2. The K^+ and Energy Requirements of the Valinomycin Stimulated pH Shift. Basic medium: 0.25 M sucrose, 3 mM succinate, 8 mM Tris · HCl, pH 7.4. Total volume 3.0 ml containing 3 mg rat liver mitochondrial equivalent protein per ml. Other additions are as indicated. The reaction was followed at 20° C with a Beckman expanded scale pH meter with a recorder attachment.

is retarded either by glutamate or phosphate, and requires further study. Acid ejection does not depend on the presence of Pi.

Direct evidence that valinomycin stimulates the active transport of K^+ by mitochondria was obtained by means of a K^+ sensitive glass electrode. The H^+ sensitive and K^+ sensitive glass electrode tracings of the valinomycin induced ion migrations, Fig. 3, show that the lowering of pH of the medium is clearly in phase with the disappearance of K^+ from the medium, i.e. the transport of K^+ into the mitochondria. Cutting off substrate derived energy with antimycin reverses both ion migrations. Calibration for the Δ potential : ΔK^+ concentratio gave figures for the ΔH^+ : ΔK^+ stoichiometry approaching 0.9 at lower K^+ concentrations (Table I). Corrections for concommitant H^+ ejection were 10 per cent of the net potential change at the cation electrode and therefore not troublesome.

Energy for the valinomycin induced ion shifts can be supplied by ATP instead of substrate. In this system oligomycin, but not antimycin, inhibits ion transport, the complete converse of the inhibitor sensitivity of substrate energized ion transport. These data, coupled with those of the previous paper (Pressman, 1964), are summarized diagrammatically in Fig. 4. The justification of the two independent Pi incorporating reactions is based on the ability of

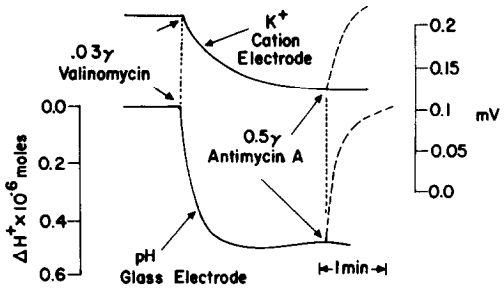

Fig. 3. Direct Measurement of Valinomycin Stimulated Uptake of K^+. K^+ disappearance from the medium was recorded by means of the Beckman Cation Electrode (No. 30947). Tracings are a composite of identical experiments measured consecutively with the cation and conventional pH electrode. Conditions same as for Fig. 2 except for supplement of 3.5 mM KCl. mV scale on right applies to the upper (K^+) tracing.

TABLE I. STOICHIOMETRY OF K^+ UPTAKE TO H^+ EJECTION

Initial Concentrations K^+	K^+ Uptake/g Protein	H^+ Ejected/g Protein	$H^+:K^+$
3.5 mM	28 μ mol	20 μ mol	.71
2.0	18	16	.89
1.0	11	10	.91

Net transport of ions measured 30 seconds after addition of .03 valinomycin. Conditions the same as in Fig. 3, except for the K^+ concentrations.

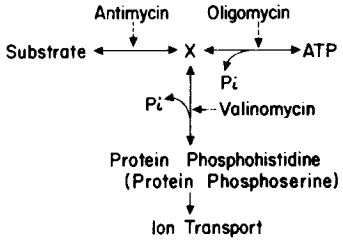

Fig. 4. Relationship of Valinomycin and Ion Transport to Mitochondrial Energy Transfer. X represents a non-phosphorylated energy intermediate common to both ion transport and ATP formation.

both DNP, and valinomycin in the presence of K^+, to cause rapid hydrolysis of mitochondrial ATP but not mitochondrial protein bound phosphate.

Brierly (1963) and Chappell et al (1963) have postulated that the H^+ ejected from mitochondria during the active transport of Ca^{++} and Mn^{++} arises

from the formation of insoluble sesquiphosphates yielding ca. one equivalent of H^+:2/3 equivalent phosphate, viz.:

$$3\ Me^{++} + 2\ H_{1\frac{1}{2}}\ PO_4^{-1\frac{1}{2}} \longrightarrow Me_3(PO_4)_2 \downarrow + 3\ H^+$$

A variation of this hypothesis is the suggested formation of hydroxyapetite (Lehninger et al, 1963).

While Pi appears necessary for optimal binding of divalent metals, it is not however a requisite for cation uptake. Chappell et al(1963) found that omission of Pi from the system raises the H^+:Mn^{++} ratio from 0.9 to 1.1, while Saris (1963) reported a similar H^+:Ca^{++} rise under equivalent conditions. The corresponding H^+:2 K^+ ratios we report in the absence of Pi, approaching 1.8 at lower K^+ concentrations, are not compatible with a phosphate precipitation hypothesis, nor is the extreme solubility of $K_3\ PO_4$.

We propose that the ejection of H^+, as well as the concomitant uptake of Pi, result from the necessity of preserving electrostatic balance during the active transport of cations. The uptake of singly charged K^+ is balanced preferentially by the complementary ejection of an equivalent of H^+. Transport by mitochondria of the singly charged guanidinium ion, which also forms no insoluble phosphates, is nevertheless accompanied by the uptake of 2/3 equivalent of Pi (Pressman, 1963 b). We conclude that the transport of divalent ions is balanced electrostatically by the ejection of one equivalent charge of H^+, and the uptake of one equivalent negative charge of Pi (at pH 7.4 $\sim$ ca. 2/3 molar equivalents). The shift of ratios for Ca^{++} and Mn^{++} resulting from omitting Pi from the medium is in qualitative if not quantitative agreement with our hypothesis. We concur with the suggestion of Saris (1963) that secondary redistribution of other ions during active transport (e.g. Na^+, Cl^-, Mg^{++}) may account for the lack of strict integral stoichiometries to obtain. We do not exclude the possibility of secondary precipitation of insoluble phosphates within the mitochondria, but strongly doubt that this represents any part of the primary transport process.

The triggering of K^+ transport by valinomycin implies the existence of a mitochondrial receptor site which, when appropriately stimulated, drastically

alters the properties of the mitochondrial ion pump. The high affinity of this site for the chemically inert valinomycin molecule suggests that this interaction is biologically purposeful. Although valinomycin has not been reported to occur in animals, the gross resemblance of its structure to that of the low molecular weight cyclic oligopeptide hormones may prove significant.

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REFERENCES

Brierley, G. P., in Energy Linked Functions of Mitochondria, Academic Press, New York, 1963, p. 237

Chance, B., and Hagihara, B., Proceedings of the Fifth International Congress of Biochemistry, Vol. V, p. 3 (1963)

Chappell, J. B., Cohn, M., and Greville, G. D., in Energy Linked Functions of Mitochondria, Academic Press, New York, 1963, p. 219

Hagihara, B., Biochim. et Biophysica Acta 46, 134 (1961)

McMurray, W., and Begg, R. W., Arch. Biochem. Biophys. 84, 546 (1959)

Lehninger, A. L., Rossi, C. S., and Greenawalt, J. W., Biochem. Biophys. Res. Comm. 10, 444 (1963)

Pressman, B. C., in Energy Linked Functions of Mitochondria, Academic Press, New York, 1963a, p. 181

Pressman, B. C., Biochem. Biophys. Res. Comm. 11, 182 (1963b)

Pressman, B. C., Biochem. Biophys. Res. Comm., preceding paper (1964)

Saris, N. E., Societas Scientiarum Fennica, Commentationes Physico-Mathematicae XXVIII 11 (1963)