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EFFECT OF A NEW MONOCARBOXYLIC ACID ANTIBIOTIC, A204, ON THE MONOVALENT CATION PERMEABILITY OF RAT LIVER MITOCHONDRIA

DAVID T. WONG, JONG-SIN HORNG and ROBERT L. HAMILL
Eli Lilly Research Laboratories, Indianapolis, Ind.

and

HENRY A. LARDY*
Institute for Enzyme Research, University of Wisconsin, Madison, Wis., U.S.A.

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Abstract—A newly isolated polyether monocarboxylic acid antibiotic, A204, is reported here to affect the translocation of monovalent cations across mitochondrial membrane. At 1 μM, A204 caused a complete inhibition of glutamate oxidation in valinomycin or monazomycin-treated rat liver mitochondria in a K^+ medium. The antibiotic has the following ion specificity: $K^+ = Rb^+ > Na^+ = Cs^+ > Li^+$. Pyridine nucleotides in intact mitochondria became oxidized upon addition of A204; however, A204 did not affect respiration supported by succinate oxidation. It also causes the leakage of K^+, transient release of H^+, and decrease of mitochondrial volume. The monovalent cation-dependent hydrolysis of ATP in valinomycin- or monazomycin-treated mitochondria was first activated and then inhibited at concentrations of A204 above 2 μM. The inhibitory effect of A204 on adenosine triphosphatase (ATPase) is also ion dependent, but it is equally supported by Na^+, K^+ and Rb^+. We can therefore classify A204 as a new ionophore with properties shared by its predecessors, monensin, nigericin and dianemycin, which induce ion permeability in biological membranes.

MONOCARBOXYLIC acid antibiotics like nigericin, dianemycin and monensin increase monovalent cation permeability of biological and artificial membranes.[1] In isolated preparations of mitochondria, these antibiotics inhibit cation uptake, the oxidation of substrate, and the ion-dependent adenosine triphosphatase (AT Pase.)[2,3] A204 is a newly discovered monocarboxylic acid antibiotic isolated from *Streptomyces albus* (NRRL 3384).[4] It is active against coccidial infection in chicks, a property shared with other monocarboxylic ionophores.[5] The physical-chemical properties of A204 suggested a possible polyether structure, and the nuclear magnetic resonance (n.m.r.) spectrum of A204 resembled that of monensin, but suggested a difference in the number of methoxyl groups.[4] The similarity in biological and chemical properties of A204 and monensin led us to examine the effect of A204 on the transport of monovalent cations in mitochondria.

MATERIALS AND METHODS

Preparation of rat liver mitochondria. Male albino rats derived from the Wistar strain, weighing about 150 g each, were obtained from a local supplier. Rat liver mitochondria were isolated and twice washed with 0·25 M sucrose.[6] Protein was determined by a modified biuret method.[7]

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Glutamate oxidation. A Clark type electrode was used to measure respiration polarographically at 30°. The medium contained: 0·17 M sucrose; 15 mM KCl; 6 mM tris-phosphate, pH 7·4; 3 mM $MgCl_2$; and mitochondrial protein of approximately 2·5 mg/ml. Monocarboxylic acid antibiotics at various amounts, 0·2 μg/ml valinomycin or 0·63 μg/ml monazomycin, and 10 mM glutamate were added in sequence at about 30 sec apart. At each concentration of A204, at least duplicate determinations were made. For ion specificity determination, KCl was replaced by an equal concentration of LiCl, NaCl, RbCl or CsCl.

Cation-dependent adenosine triphosphatase measurement. At a final volume of 1 ml, the incubation mixture contained: 0·225 M sucrose; 30 mM tris–HCl, pH 7·4; 20 mM tris-acetate, pH 7·4; 30 mM monovalent cation chloride; and 6 mM ATP-tris salt at pH 7·4. Either valinomycin, 0·1 μg/ml, or monazomycin, 15 μg/ml, was used as an inducing agent. Incubation was initiated by adding 1 mg protein of mitochondria at 30° for 10 min. Trichloroacetic acid at 5% was used to terminate the reaction, and the extract, after centrifugation, was used for inorganic phosphate determination according to the method of Fiske and Subbarow.[8]

Simultaneous measurements of oxygen consumption, H^+ and K^+ concentrations, and light scattering. These measurements were carried out with the apparatus designed by Pressman.[9] In a final volume of 5 ml, the reaction mixture consisted of: 0·2 M sucrose; 6 mM KCl; 4 mM triethanolamine (TEA) phosphate, pH 7·4; 3 mM $MgCl_2$; 12 mM succinate (TEA), pH 7·4; 10^{-6} M rotenone; and 9·5 mg of mitochondrial protein.

Simultaneous measurement of respiration and redox-levels of pyridine nucleotides in intact mitochondria. Oxygen consumption was measured with a vibrating oxygen electrode purchased from American Instrument Company. Redox levels of pyridine nucleotides were monitored by a fluorometer attachment to an Aminco-Chance dual wavelength, split-beam spectrophotometer with appropriate filters for excitation at 366 nm and emission at >405 nm. The reaction mixture at 25° consisted of 0·25 M sucrose, 10 mM KCl, 10 mM potassium phosphate at pH 7·4, and mitochondrial protein at 1·5 mg/ml in a total volume of 3 ml. Subsequently, 10 μmoles malate, 10 μmoles glutamate, 2 μg valinomycin and 6 μg A204 were added as indicated. The increase of fluorescence denoted reduction of pyridine nucleotides.

Simultaneous measurement of mitochondrial volume changes and 8-anilino-naphthalene sulfonic acid (ANS) fluorescence in intact mitochondria. Mitochondrial volume changes were detected at 520 nm by the split-beam setting of the Aminco-Chance dual wavelength, split-beam spectrophotometer, and the fluorescence of ANS was monitored by using a fluorometer attachment with appropriate filters for an excitation at 366 nm and emission at >450 nm. The fluorescence changes observed were not due to the redox changes of pyridine nucleotides because mitochondria were pretreated with 13 μM rotenone. The reaction mixture at 25° contained: 0·25 M sucrose; 10 mM KCl; 10 mM tris-phosphate, pH 7·4; mitochondria, 0·92 mg/ml; and 13 μM rotenone in a total volume of 6 ml. ANS in ethanol solution was then added to the suspension at 83 μM. The mixture was then divided into two cuvettes. Subsequently, reagents were added to the reference cuvette which was facing the attached fluorometer. The following reagents were added as indicated: 5 μmoles ATP-Mg, 2·5 μg valinomycin, 10 μg A204, 5 μg oligomycin, 5 μM carbonyl cyanide (*m*-chlorophenyl hydrazine, m-Cl-CCP; Calbiochem), and 6·6 mM succinate-tris, pH 7·4.

Antibiotics. Valinomycin, monazomycin and oligomycin were crystalline materials

isolated by our company. Rotenone was obtained from S. B. Penick & Company. All other biochemicals were purchased from Sigma.

RESULTS

The enhancement of mitochondrial metabolism by potassium was recognized as early as 1952 by Pressman and Lardy.[10] It was not until the discovery of ionophorous agents such as gramicidin, valinomycin and monazomycin that the striking capacity of mitochondria to take up monovalent cations upon the expenditure of respiratory energy was realized.[11] The reported uncoupling effects[12] of these agents arise from their stimulation of cyclic and steady state cation transport.

Such an effect of valinomycin on respiration of rat liver mitochondria is shown in Fig. 1. In these experiments, both respiration and the redox levels of pyridine nucleotides inside mitochondria were monitored. In experiment A, state 4 respiration of

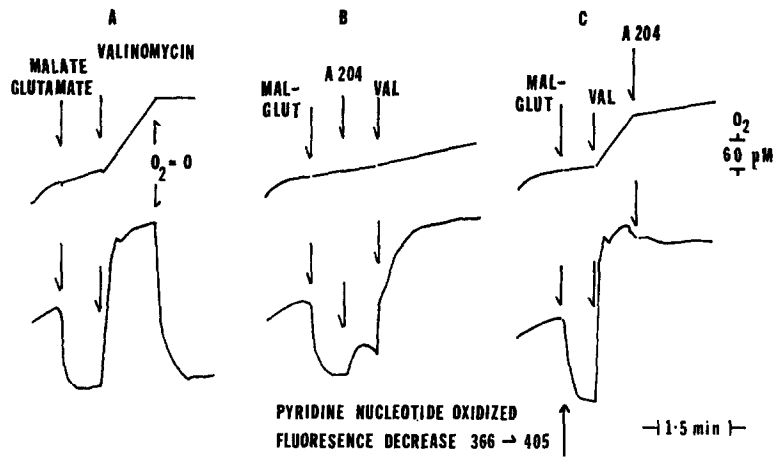

FIG. 1. Effect of valinomycin and A204 on oxygen consumption and redox levels of pyridine nucleotides in intact rat liver mitochondria. The upward deflection of the O_2 trace represents disappearance of oxygen in medium. The downward deflection of the lower trace indicates reduction of mitochondrial pyridine nucleotides. Experimental detail was described in Materials and Methods.

mitochondria was relatively slow, even in the presence of substrate, malate-glutamate. The addition of valinomycin caused a 6-fold increase in respiratory rate which continued until the exhaustion of oxygen. During this sequence of additions, the redox states of pyridine nucleotides were subject to cyclic changes. The addition of malate-glutamate induced a complete reduction, whereas the addition of valinomycin caused an oxidation exceeding the initial level followed by a return to the fully reduced level when oxygen was exhausted. When A204 was added prior to valinomycin (experiment B), the latter agent lost the ability to stimulate respiration and the pyridine nucleotides remained in an oxidized state. When A204 was added after valinomycin (experiment C), the activated rate of respiration was terminated and the pyridine nucleotides were maintained in an oxidized state. Thus, A204 blocked the valinomycin-stimulated oxygen uptake of mitochondria.

The effect of A204 on the oxidation of glutamate was then examined. In KCl medium, valinomycin-induced oxygen uptake was usually higher than that induced by

monazomycin. As the concentration of A204 increased, respiration activated by either inducer fell and reached about the same end point (Fig. 2a). The degree of inhibition in both cases was almost identical at each concentration of A204 (Fig. 2b).

The inhibition of glutamate oxidation depended on the monovalent cation used in the medium (Fig. 3). A204 inhibited most strongly with medium containing K^+ or Rb^+ in either monazomycin-treated (left panel) or valinomycin-treated (right panel) mitochondria. When monazomycin was the inducer A204 imposed a 40 per cent inhibition

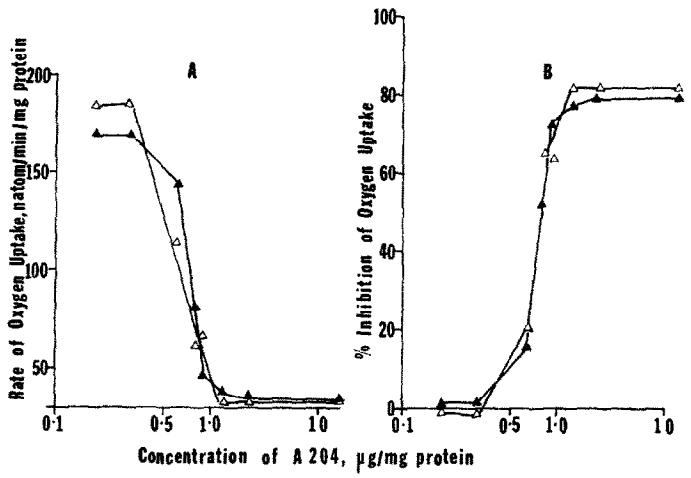

FIG. 2. Inhibition of glutamate oxidation by A204. (A) Rate of oxygen uptake was plotted against concentration of A204. (B) Same result was expressed as percentage of inhibition. Experimental conditions were described in Materials and Methods.

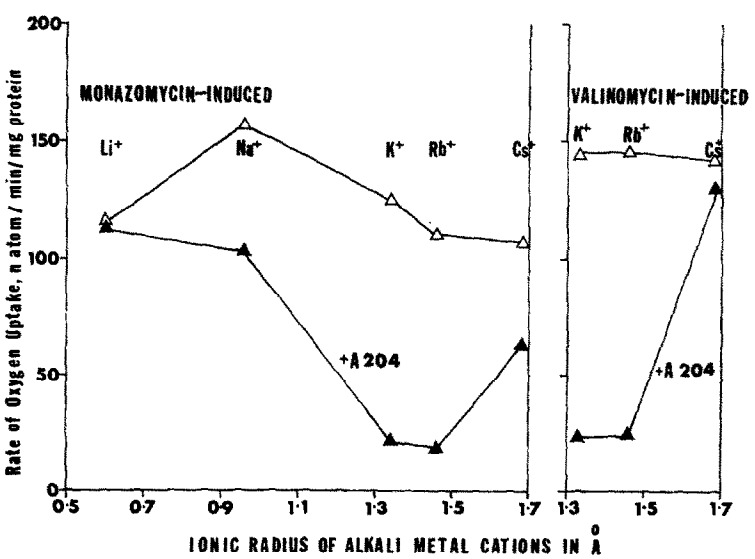

FIG. 3. Inhibition of glutamate oxidation by A204 in the presence of various monovalent cations. Experimental conditions were described in Materials and Methods.

in medium containing Na^+ or Cs^+. However, A204 did not inhibit oxygen uptake in Cs^+ medium when valinomycin was the inducer. The difference of A204 action in Cs^+ medium probably originated from some unknown difference of the two inducing agents. A204 was least active in Li^+ medium. These results were presented as percentage of inhibition as shown in Fig. 4.

The action of A204 was further examined by the simultaneous measurements of oxygen uptake, cation movements and light scattering of mitochondria (Fig. 5). As seen earlier, valinomycin induced oxygen consumption and swelling of mitochondria as it caused the withdrawal of K^+ from the medium and the extrusion of H^+ into the medium (left panel). After the mitochondrial cations reached a new steady state, 5 μg/ml of A204 reversed the K^+ movement and caused contraction of volume (middle panel). At a final concentration of 10 μg/ml A204 reversed both traces only half way.

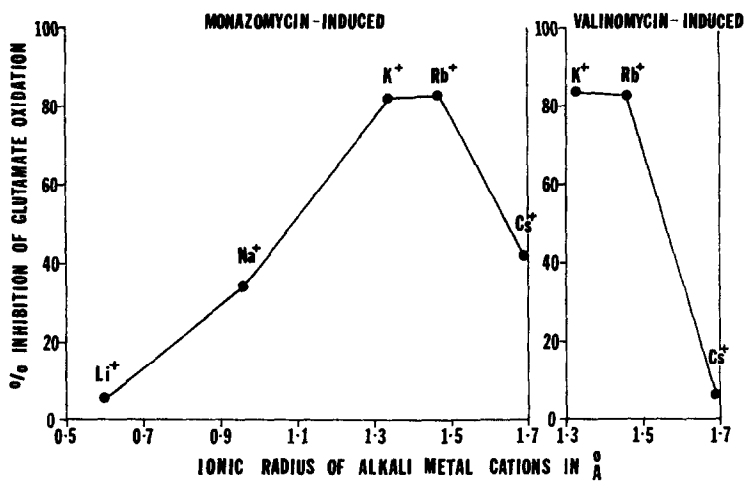

FIG. 4. Ion specificity of A204 in inhibition of glutamate oxidation. Data obtained from Fig. 3

However, at each addition of A204, H^+ was first released and was then slowly taken up by the mitochondria. The residual amount of K^+ was released with additional influx of H^+ when 0·2 μg/ml of nigericin was added. At this point, contraction of mitochondrial volume was complete. The sequence of events was reversed when A204 was added before valinomycin (right panel). In all three cases, the succinate-supported respiration continued at accelerated rates after the addition of valinomycin and was not affected by A204. This is consistent with the effect of other monocarboxylic ionophores which only inhibited oxygen uptake with glutamate, α-ketoglutarate or malate as substrate.[3]

Valinomycin induces the hydrolysis of ATP as a result of its ability to facilitate active cation uptake into the mitochondria. Figure 6 shows that A204 potentiated that effect at low concentration and became inhibitory as the concentration was increased beyond 2 μg/mg of mitochondrial protein. A204 itself induced some hydrolysis of ATP.

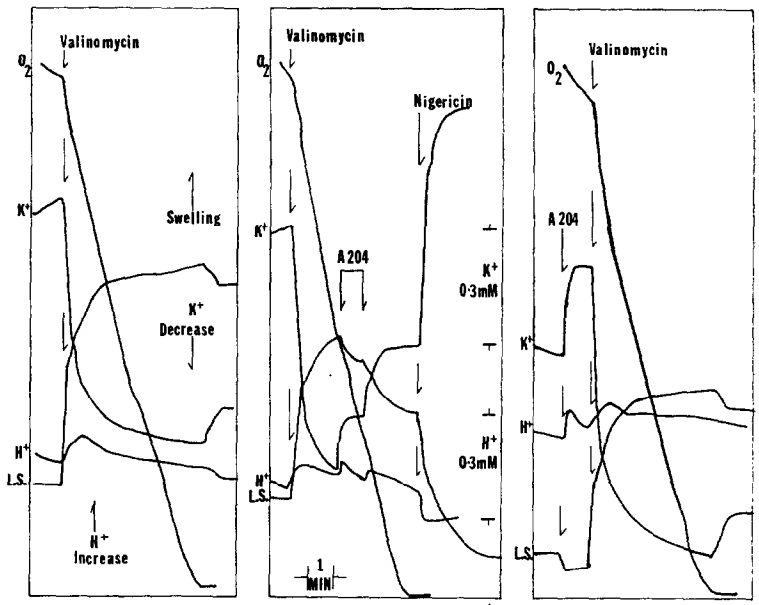

FIG. 5. Effect of valinomycin and A204 on H^+ and K^+ transport, light-scattering changes, and oxygen consumption supported by succinate. A downward deflection of the K^+ trace represents a decrease of its concentration in the medium or uptake of K^+ by the mitochondria. An upward deflection of the H^+ trace represents an increase of its concentration in the medium. An upward deflection of light scattering (L.S.) is associated with swelling of the mitochondria. The indicated additions were: 10^{-7} M valinomycin, 25 μg A204, twice (middle panel), or 50 μg A204 (right panel) and 1 μg nigericin. Other conditions were described in Materials and Methods.

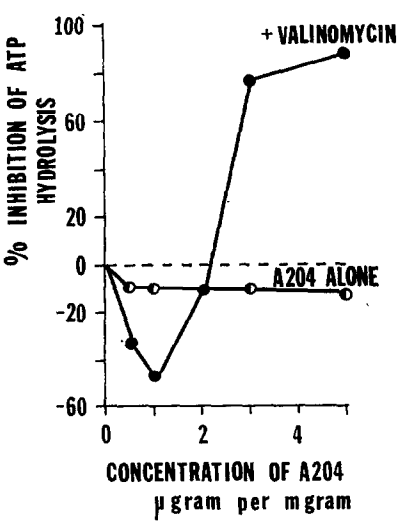

FIG. 6. Effect of A204 on the hydrolysis of ATP in mitochondria. In the absence of A204, the control rate of ATP hydrolysis was 0·68 μmole/mg of protein and the valinomycin-induced rate was 2·51 μmoles/mg of protein. Experimental conditions were described in Materials and Methods.

The inhibition of ATP hydrolysis also depended on the cation species used in the incubation medium (Fig. 7). A204 at 3 μg/mg of protein inhibited most when the cation was Na^+, K^+ or Rb^+. The effect was less with Cs^+, and no effect was found in medium containing Li^+.

The antibiotic A204 also caused shrinkage of mitochondrial volume and changes in membrane conformation. In Fig. 8, the upper traces represent volume changes and the

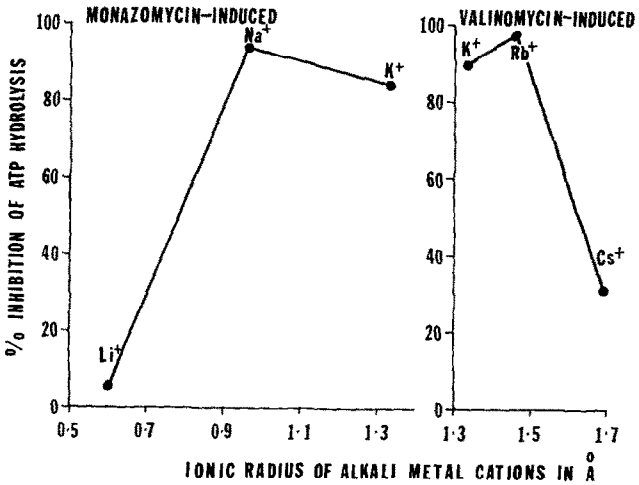

FIG. 7. Ion specificity of A204 in inhibition of the ion-dependent adenosine triphosphatase. A204 at 3 μg/ml and inducer, 0·1 μg/ml of valinomycin or 15 μg/ml of monazomycin, were used. Experimental conditions were described in Materials and Methods.

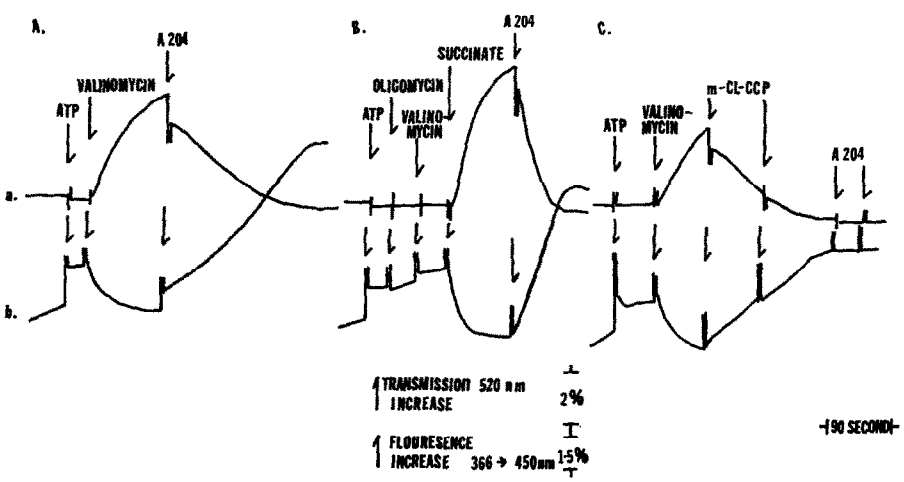

FIG. 8. Effect of A204 on mitochondrial volume changes and membrane conformational changes as indicated by 8-anilino-naphthalene sulfonic acid (ANS) fluorescence. The upward deflection of the upper trace (transmission at 520 nm) represents an increase in mitochondrial volume. The lower trace represents conformational changes of the mitochondrial membrane as indicated by ANS fluorescence. Experimental conditions were described in Materials and Methods.

bottom traces show the changes of fluorescence of 8-anilino-naphthalene sulfonic acid (ANS), a probe which senses conformational changes of the mitochondrial membrane.[13] In the presence of ATP, no volume changes took place, although ATP induced additional binding of ANS. The addition of valinomycin induced an increase in transmission of light, suggesting an increase in mitochondrial volume. The subsequent addition of A204 caused a decrease in light transmission due to shrinkage of mitochondria. The fluorescence of ANS was first decreased with valinomycin and then increased with A204, indicating the unfolding and folding of the membranes respectively.

The valinomycin-induced swelling and membrane conformation of mitochondria depended on either ATP hydrolysis, which was oligomycin-sensitive, or respiratory energy by the oxidation of succinate (experiment B). These processes were also sensitive to an uncoupling agent, m-Cl-CCP (experiment C). A204 exerted an effect on the membrane only when mitochondria were swollen by an earlier addition of valinomycin (compare experiments A and B with C).

DISCUSSION

A204 inhibited the mitochondrial cation pump which was induced either with valinomycin or monazomycin. Consequently the cation-dependent adenosine triphosphatase was inhibited. Associated with the inhibition of cation uptake, A204 also caused shrinkage of mitochondria. These properties were shared with nigericin, dianemycin and monensin.

The extensive number of carboxylate ionophores now known makes it possible to construct meaningful structure and ion specificity for comparisons. Antibiotics with a higher molecular weight tend to have a higher limit in the size of cations with which the antibiotic exhibits activity. Monensin (670) is most active against Na^+, nigericin (736) against K^+, and A204 (960) against Rb^+ and Cs^+ to a lesser degree. Dianemycin (958) is an exception because it does not discriminate between any alkali metal cations;[14] perhaps the molecule of dianemycin is a more flexible one. A comparative study on the molecular structures of both A204 and dianemycin would be informative in this regard.

The question of the primacy of cation transport over substrate transport in mitochondria has been raised.[15] The experiments, as illustrated in Fig. 1, might serve to provide insight into this question. When valinomycin stimulated respiration in mitochondria, it also caused an oxidation of pyridine nucleotides (experiment A). The oxidized state of the coenzymes was maintained even when respiration was inhibited by A204 (experiment C). Apparently the extra mitochondrial malate and glutamate were not available to donate electrons for the respiratory chain. However, when only A204 was added (experiment B), the fully reduced coenzymes were slightly oxidized. The subsequent addition of valinomycin slowly oxidized the coenzymes to the maximum level. The kinetic patterns seem to suggest that valinomycin induced cation transport, which in turn activated respiration to its upper limit. The availability of substrates into mitochondria would not keep pace with the demand and kept the coenzymes in a more oxidized level. The findings are therefore consistent with the idea that cation transport has led to the secondary entry of substrates into the mitochondria.[15,16]

REFERENCES

1. B. C. Pressman, *Fedn Proc.* **27**, 1283 (1968).
2. S. N. Graven, S. Estrada-O and H. A. Lardy, *Proc. natn. Acad. Sci. U.S.A.* **56**, 654 (1966).
3. S. Estrada-O, B. Rightmire and H. A. Lardy, *Antimicrob. Agents Chemother.* **1967**, 279 (1968).
4. R. L. Hamill, M. M. Hoehn and M. Gorman, *Antimicrob. Agents Chemother. Abstr.* **15**, 7 (1970).
5. R. F. Shumard and M. E. Callender, *Antimicrob. Agents Chemother.* **1967**, 369 (1968).
6. W. C. Schneider and G. H. Hogeboom, *J. biol. Chem.* **183**, 123 (1950).
7. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
8. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).
9. B. C. Pressman, in *Methods in Enzymology* (Eds. R. W. Estabrook and M. E. Pullman), Vol. 10, p. 714. Academic Press, New York (1967).
10. B. C. Pressman and H. A. Lardy, *J. biol. Chem.* **197**, 547 (1952).
11. C. Moore and B. C. Pressman, *Biochem. biophys. Res. Commun.* **15**, 562 (1964).
12. W. McMurray and R. W. Begg, *Archs Biochem. Biophys.* **84**, 546 (1959).
13. A. Azzi, B. Chance, G. K. Radda and C. P. Lee, *Proc. natn. Acad. Sci. U.S.A.* **62**, 612 (1969).
14. P. J. F. Henderson, J. D. McGivan and J. B. Chappell, *Biochem. J.* **111**, 521 (1969).
15. E. J. Harris, M. P. Höfer and B. C. Pressman, *Biochemistry, N.Y.* **6**, 1348 (1967).
16. H. A. Lardy and S. M. Ferguson, *A. Rev. Biochem.* **38**, 991 (1969).