# EFFECT OF ION-TRANSPORTING ANTIBIOTICS ON THE ENERGY-LINKED REACTIONS OF SUBMITOCHONDRIAL PARTICLES*

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Certain antibiotics have the property of activating ion movements across natural and artificial membranes (1). They have been utilized as a tool in the elucidation of the relationship between membrane phenomena and energy coupling mechanisms in mitochondria (1) in chloroplasts (2) and chromatophores (3,4,5).

Submitochondrial particles derived by sonic disruption of mitochondria are still capable of respiratory-chain-linked energy coupling and oxidative phosphorylation, and are a more elementary system in which to understand the relationship between energy coupling and ion transport across the inner mitochondrial membrane.

The data reported in this paper show that ion-transporting antibiotics of the valinomycin and nigericin type strongly affect the energy-linked reactions of submitochondrial particles. The results are discussed in terms of the current hypotheses of energy coupling in oxidative and photosynthetic phosphorylation.

## MATERIALS AND METHODS

EDTA particles derived from beef heart mitochondria were prepared as described previously (6). Oxygen consumption was measured polarographically

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with a Clark oxygen electrode. The energy-linked pyridine nucleotide transhydrogenation (7) and the ATP-supported reduction of $NAD^+$ by succinate (8) were assayed according to Ernster and Lee. The energy-linked BTB† and ANS responses were measured as described by Chance and Mela (9) and by Azzi, et al. (10), respectively. Valinomycin and nigericin were kindly supplied by Dr. B. C. Pressman of this laboratory.

## RESULTS

Lee and Ernster (11) have shown that oligomycin induces an inhibition of respiration in E-SMP, which is released by uncouplers; furthermore, in the presence of oligomycin there is a stimulation of the respiratory chain supported energy-linked transhydrogenase reaction and of the energy-linked reduction of $NAD^+$ by succinate, which are also inhibited by uncouplers. In Fig. 1, the release of the oligomycin-induced respiratory control is plotted as a function of KCl concentration. Like other uncouplers, the combination of valinomycin and nigericin released the oligomycin control, although valinomycin alone had no effect, and nigericin alone released about 50% of the control. Furthermore, addition of FCCP to the valinomycin- and/or nigericin-treated system resulted in a further stimulation of respiration by about 50%, in comparison to that induced by either alone. The question arises as to whether these antibiotics are acting at the level of the electron transfer carrier or the energy transfer process. In order to elucidate this question, other energy-linked reactions were studied.

Figure 2 shows the effect of the antibiotics on the energy-linked pyridine nucleotide transhydrogenase reaction. As can be seen, the presence of both antibiotics leads to 95% inhibition of the rate of NADPH formation, while nigericin alone in the presence of $K^+$ has no effect, and valinomycin alone in the presence of $K^+$ inhibits about 50%. There is no effect of the

†Abbreviations used: BTB, bromthymol blue, 3,3" dibromothymolsulfonphthalein; E-SMP, EDTA submitochondrial particles; ANS, N-arylaminonaphthalene sulfonate; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

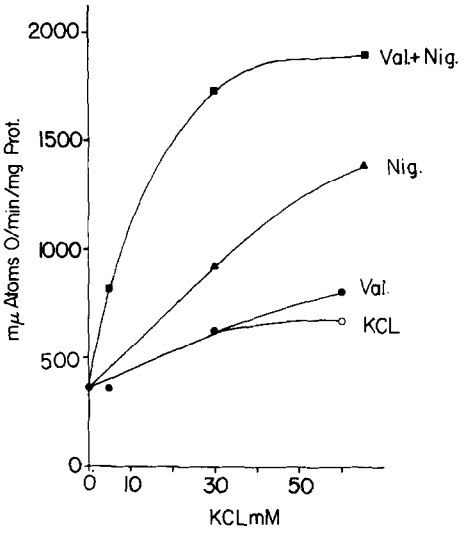


Figure 1. Effect of valinomycin and nigericin on the oligomycin-induced respiratory control as a function of KCl concentration. The reaction mixture contained 0.1 mg protein per ml of E-SMP, 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 0.6 μM valinomycin, and 0.06 μM nigericin. Final volume, 3 ml. Temperature, 30° C.

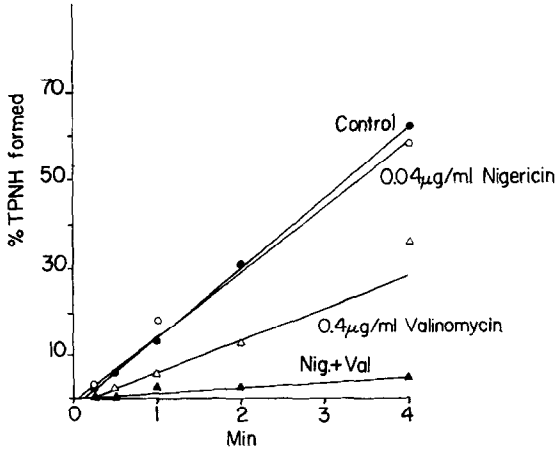


Figure 2. Effect of valinomycin and nigericin on the energy-linked pyridine nucleotide transhydrogenation. The reaction mixture contained 0.1 mg protein per ml of E-SMP, 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4, 0.05 M KCl, 0.5 μg per ml of oligomycin, 3.3 μM rotenone, 135 μM NADH, 5 mM succinate. Final volume, 10 ml. Temperature, 30° C. The reaction was started by the addition of 180 μM NADP. Assays were done according to the method of Lee and Ernster (7).

antibiotics on the non energy-linked reaction.

BTB (9,12), and more recently, ANS (10), have been shown to act as indicators of the energized state of submitochondrial particles. Figure 3 shows the effect of the antibiotics on the energy-linked BTB (lower trace) and ANS (upper trace) responses. 3A shows, as a control, the cycle of oxidation of 100 μM NADH. 3B is a cycle of NADH oxidation in the presence of 20 mM KCl; thereafter, 70 nM nigericin was added. 3C shows that the cycles are now approximately 30% larger in amplitude and 50% shorter in duration. In 3D, after the addition of valinomycin, the cycle is 95% inhibited; this inhibition is comparable to that induced by FCCP under similar conditions. The effect of valinomycin alone in the presence of $K^+$ (not shown) is to inhibit the BTB and ANS responses only about 50%.

The effect of the antibiotics on the energy-linked reduction of $NAD^+$ by succinate was also studied. Valinomycin and nigericin in the presence of KCl inhibited 100% of the reaction, whereas nigericin alone in the presence of $K^+$ had no effect, and valinomycin alone in the presence of $K^+$ inhibited only 50%.

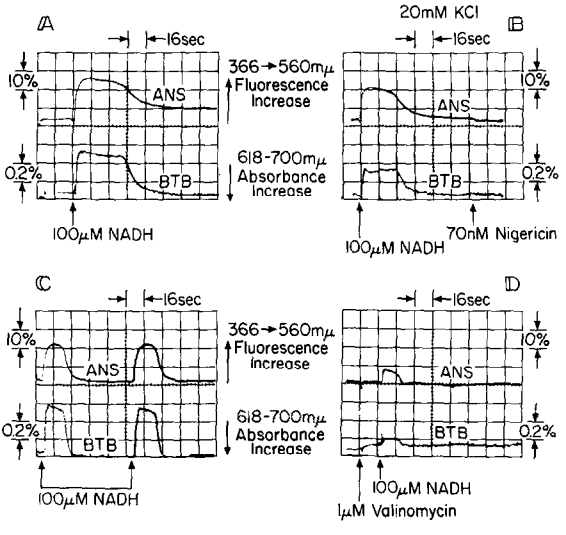


Figure 3. Effect of nigericin and valinomycin on the energy-linked BTB and ANS responses. 0.9 mg protein per ml of E-SMP in 0.3 M mannitol-sucrose, 20 mM Tris-HCl, pH 7.4, supplemented with 0.5 μg per ml of oligomycin, 5 μM BTB, and 10 μM ANS. Final volume, 1 ml. Temperature, 25° C.

The well-documented specificity of valinomycin to $K^+$ rather than to $Na^+$ (1,13) is demonstrated by the complete lack of inhibition of all the energy-linked reactions in a medium in which the $K^+$ has been replaced by $Na^+$. Nigericin was partially effective in a $Na^+$ medium, according to its known specificity (1). The effect observed on addition of both antibiotics was strictly dependent on the presence of $K^+$.

## DISCUSSION

Previous studies have considered the requirement of a cation (tentatively identified with $Ca^{++}$) movement opposite to the $H^+$ uptake during coupled respiration (9). The succinate supported inward movement of $Ca^{++}$ observed by Loyter and Racker (14) in submitochondrial particles is inconsistent with the inward direction of the $H^+$ movements described by Chance and Mela (9) and Mitchell and Moyle (18); the cation pumps seem to operate in opposite directions.

The results reported here afford indirect evidence that the cation required for $H^+$ movements in submitochondrial particles (9,18) may be $K^+$, and direct evidence of an interaction between monovalent cation movements and the process of electron transfer and energy conservation in submitochondrial particles.

The stimulation by nigericin and $K^+$ of the rate of oxygen uptake in an uncoupled system points in fact to an influence of the ionic environment of the respiratory chain on the rate of electron transport. Due to the known $K^+/H^+$ exchange promoted by nigericin, it is evident that the presence of either a high $K^+$ or low $H^+$ concentration is a requirement for having a maximal rate of respiration.

The effect of valinomycin and $K^+$ and the synergistic effect of valinomycin and nigericin in the presence of $K^+$, inhibiting several energy-linked reactions in submitochondrial particles, suggests as well a close

interaction between ion movements and energy conservation.

One mechanism is based upon the above mentioned valinomycin-activated outward movement of $K^+$ which is responsible for the inhibition of the transhydrogenase, the BTB-ANS responses and the reversal of electron transfer.

The combined effects of valinomycin and nigericin suggest a cyclic energy dissipating ion movement across the membrane, in which the effect of nigericin would be to restore the $K^+$ content inside the vesicle depleted by the activity of a pump or of a membrane potential, that operates in the presence of valinomycin, as proposed for mitochondria (1) and chromatophores (3,5,16,17).

An additional explanation is that the combined effect of the ion carrying antibiotics is to transport ions into regions of low dielectric constant of the membrane, which are required for energy coupling. In this case, uncoupling would result not by cyclic transport, but due to the simple binding of ionic species in such an environment. This is supported by the inhibitory effect of valinomycin and nigericin in the presence of $K^+$ on the conformational changes indicated by ANS and BTB.

## SUMMARY

The effects of ion-transporting antibiotics on the energy-linked reactions of submitochondrial particles were studied. It was found that valinomycin and nigericin in the presence of $K^+$ led to the release of the oligomycin-induced respiratory control, and to a virtually complete inhibition of the four energy-linked reactions: pyridine nucleotide transhydrogenation, BTB and ANS responses, and reversal of electron transfer. Valinomycin alone in the presence of $K^+$ did not release the oligomycin-induced control, but inhibited the energy-linked reactions by approximately 50%. Nigericin alone in the presence of $K^+$ stimulated the oligomycin-inhibited respiration by approximately 50%, but did not affect the energy-linked reactions. The results are discussed in terms of current hypotheses of energy coupling in oxidative and photosynthetic phosphorylation.

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