*ANTIBIOTIC-MEDIATED TRANSPORT OF ALKALI IONS ACROSS LIPID BARRIERS**

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Investigations of the effects of valinomycin on mitochondria[1, 2] led to the discovery of antibiotic-mediated ion transport.[3] Subsequent work revealed that this property is shared by valinomycin, the enniatins, the macrolide actins, and the gramicidins, including an extensive array of natural and synthetic analogues.[4–6] Passive ion permeability effects have been reported obtained with these agents on other systems including erythrocytes,[7] synthetic lipid vesicles,[7] and artificial bimolecular lipid leaflets.[8, 9] The macrolide actins can even mediate cation transport, as detected by biionic potential development across a bulk phase of CCl_4.[10] All these antibiotics, which have in common low molecular weights (*ca.* 500–1500), a curious alternation of D and L configurations, lipid solubility, and a lack of ionizable groups, will be referred to collectively as the *valinomycin class* of antibiotics.

A second class of transport-mediating antibiotics, including nigericin dianemycin, and others, has been found to reverse transport induced by the valinomycin class.[11–14] All members of the second class possess low molecular weights (450–950), and lipid solubility, and contain an ionizable carboxyl group.[14] This communication will establish that both classes of antibiotics act analogously in many respects, both in mitochondria and in a variety of other lipid barrier systems.

Methods and Materials.—Multiparameter measurements of rat liver mitochondria and other systems, during antibiotic-induced ion movements, were carried out with the apparatus described in detail previously.[15] K^+ was monitored with the Beckman 39047 electrode, O_2 by a Clark-type membrane electrode, pH by the A. H. Thomas 4858 combination electrode, fluorescence by 450-mμ light excited by a 366-mμ beam, and light-scattering at 650 mμ. Mitochondria were prepared and protein was determined as described previously.[16] Samples of nigericin were obtained from H. A. Lardy, R. Harned (Commercial Solvents Corp.), and M. Gorman (Eli Lilly Co.), dianemycin from Lardy and Gorman, oligomycin from F. M. Strong, and p-trifluoromethoxycarboxylcyanide phenylhydrazone (FCCP) from P. Heytler (duPont). Valinomycin was prepared by means of a *Streptomyces* culture donated by J. C. McDonald according to a modification of his procedure.[17]

Results.—The responses of mitochondria to valinomycin addition as shown in Figure 1 have been detailed previously.[4] A subsequent addition of nigericin induces a rapid discharge of K^+ accompanied by a countermovement of H^+ and an increase in light-scattering, indicative of mitochondrial contraction, as reported by Graven *et al.*[11] Under the conditions of Figure 1*A*, respiration is stimulated initially signifying an increased energy load, before becoming strongly inhibited as the loss of K^+ progresses. In these experiments the concentration of dissolved oxygen does not become rate limiting. The transient stimulation of respiration was presumably not apparent in previous work because of the presence of phosphate in the medium, which increases respiration in the presence of valinomycin-type antibiotics[3] thereby obscuring any subsequent stimulation by nigericin.

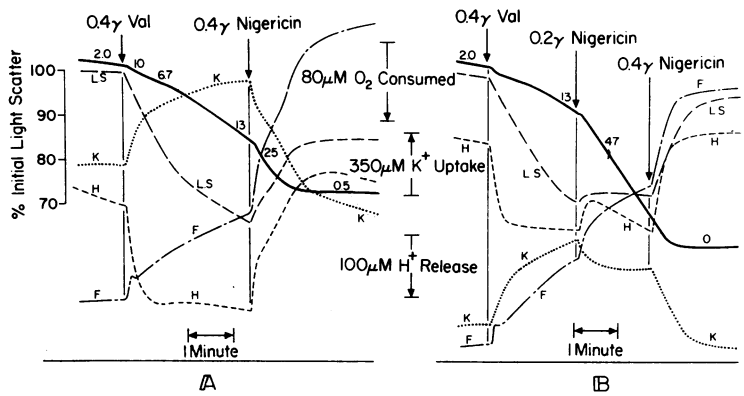

FIG. 1.—Effect of nigericin on mitochondrial respiration following valinomycin addition. In expt. *A* the nigericin dose (0.4 γ) was larger than the initial dose (0.2 γ) of expt. *B*. The system contained: 3 mM Tris glutamate; 3 mM Tris malate; 10 mM Tris Cl; 5 mM KCl; 200 mM sucrose; 2.4 mg mitochondrial protein/ml; final volume, 10 ml; pH 7.4; T = 22°. Antibiotics valinomycin (*Val*) and nigericin were added in EtOH. Identification of traces: *L.S.*, light scattering; *H*, H^+; *K*, K^+; *F*, fluorescence (upward deflection indicates decrease, i.e., pyridine nucleotide oxidation); O_2 (*solid line*) identified by adjacent figures representing μmoles O_2 consumption/min/gm protein at a given region.

The respiratory inhibition in mitochondria by either nigericin[11–14] or uncoupling agents has been related to the impeded accumulation of substrates within the mitochondria[18] rather than intrinsic inhibition of dehydrogenases.[19] This is in accord with the fluorescence tracing of Figure 1*A* indicating an extensive oxidation of pyridine nucleotide upon nigericin addition, as would be expected with impaired substrate availability. These data are also consistent with the previous observation that all the respiratory carriers, including the DPNH primarily responsible for the fluorescence signal, go oxidized during uncoupler-induced respiratory inhibition.[20] If less nigericin is added (Fig. 1*B*), so that the loss of K^+ and fluorescence are less pronounced, the resultant stimulation of respiration is sustained until a second addition of nigericin completes the discharge of K^+. The stimulatory phase of respiration can be attributed to the increased nigericin-induced passive release of K^+ which permits the valinomycin-induced, energy-utilizing uptake of K^+ to proceed more rapidly. These effects of nigericin are paralleled by dianemycin.

Another indication that the primary effect of nigericin does not involve a mitochondrial energy transfer process is that the antibiotic initiates a rapid discharge of K^+ from mitochondria whose energy sources are blocked by both rotenone (endogenous substrate inhibitor) and oligomycin (endogenous adenosine 5′-triphosphate inhibitor) (Fig. 2*A*). The endogenous respiration block by rotenone is evident in the oxygen trace. Since the measured K^+ permeability of untreated mitochondria is low, particularly when depleted of energy,[21] the K^+ discharge produced by nigericin must indicate an increase in K^+ permeability.

Valinomycin, which also increases K^+ permeability[21] fails to induce a comparable K^+ loss unless added in conjunction with an uncoupler, e.g., FCCP (Fig. 2*B*, *C*).

Figure 3 demonstrates that the ability of nigericin to raise K^+ permeability is not

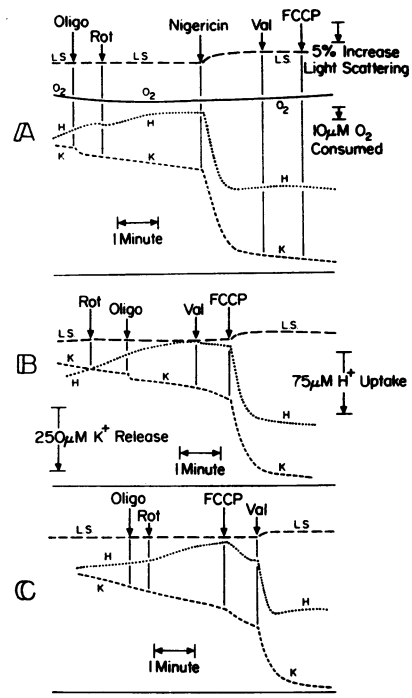

FIG. 2.—Effect of various agents on the K^+ for H^+ exchange in energy depleted mitochondria. The system contained: 10 mM Tris Cl; 5 mM KCl; 225 mM sucrose; 2.7 mg protein/ml; pH 7.25, T = 22°. Antibiotic additions at indicated points: 25 γ oligomycin (*Oligo*); 5 γ rotenone (*Rot*); 0.4 γ nigericin; 0.4 γ valinomycin (*Val*). Final concentration p-trifluoromethoxycarboxylcyanide phenylhydrazone (*FCCP*) when added, 10^{-7} *M*. Identical sensitivity calibrations apply to each experiment.

exclusive to mitochondria. In Figure 3*A* nigericin induces a downhill K^+ for H^+ exchange across the dog erythrocyte membrane. Unrecorded movements of Na^+ or Cl^- presumably account for the lack of equivalence of the observed ΔK^+ and ΔH^+. With human erythrocytes, which contain high K^+ and low Na^+, the ΔK^+ and ΔH^+ are more nearly equivalent. In Figure 3*B*, a similar result is obtained with vesicular microsomes obtained from rat brain.[22] Prior addition of dinitrophenol (DNP) and valinomycin ensures that the subsequent K^+ for H^+ exchange induced by nigercin is not due to mitochondrial contamination.

The apparent effect of nigericin on the K^+ permeability of lipid membrane systems, and its known chemical properties as a lipid-soluble carboxylic acid,[14, 23] suggested that it could carry alkali ions across a lipid barrier as the lipid-soluble

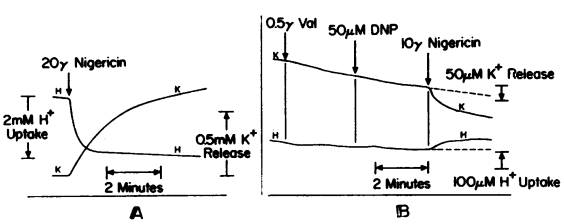

FIG. 3.—Effect of nigericin on nonmitochondrial systems.

Expt. A, performed by Mr. D. Rosenfeld, employed 78 mg dry wt/ml dog erythrocytes (twice washed in 250 mM sucrose-5 mM Tris Cl, pH 7.4). The medium contained 10 mM NaCl; 3.5 mM Tris Cl, pH 7.4; 250 mM sucrose. The erythrocytes, comprising *ca.* 25% of the volume of the system contained 121 mM Na^+ and 4.3 mM K^+.

Expt. B employed rat brain microsomes (2.8 mg protein/ml), 17 mM Tris Cl, pH 7.4, and 250 mM sucrose. The total K^+ in this system was 0.9 mM.

complex of the dissociated species. Protons would then move in the counter-direction as the undissociated carboxylic acid. Accordingly, we observed that nigericin and dianemycin can abstract alkali ions from an aqueous phase into a less polar solvent such as butanol-toluene (Fig. 4). Complexing with $^{86}Rb^+$ increased strikingly at high pH for nigericin (Fig. 4*A*) and dianemycin (Fig. 4*B*) indicating that their dissociation aids complex formation. From the ability of the unlabeled alkali ions to compete with $^{86}Rb^+$ for complexing with the antibiotics, preliminary results indicate the order of affinities at pH 10.4 to be: for nigericin, $K^+ > Rb^+ > Na^+ \gg Cs$; for dianemycin, $K^+ = Na^+ = Rb^+ > Cs^+$. Since K^+, Rb^+, and Cs^+ all exhibit similar Stokes' radii in aqueous solution and Na^+ a somewhat larger one,[24] it is difficult to account for the complexing properties of these antibiotics by a simple molecular fit with either the hydrated or unhydrated alkali ion species (refs. 8, 14, 25, 26, but cf. also ref. 4).

Complex formation between valinomycin and alkali ions was also detected. In this case the entry of the complex into the nonaqueous phase is not greatly enhanced at high pH in keeping with the lack of dissociable groups in valinomycin. On the other hand, one would expect the entry of the net positively charged complex into the nonaqueous phase to be favored by a suitable lipophilic counterion. In Figure 4*C*, 10 mM laurate fulfills this function. Since the nigericin-Rb^+ and the dianemycin-Rb^+ complexes exist in this system presumably as ion pairs without net charge, laurate does not favor their formation within the nonaqueous phase. Lardy *et al.* have also referred to the complexing properties of the ion-carrying antibiotics.[14]

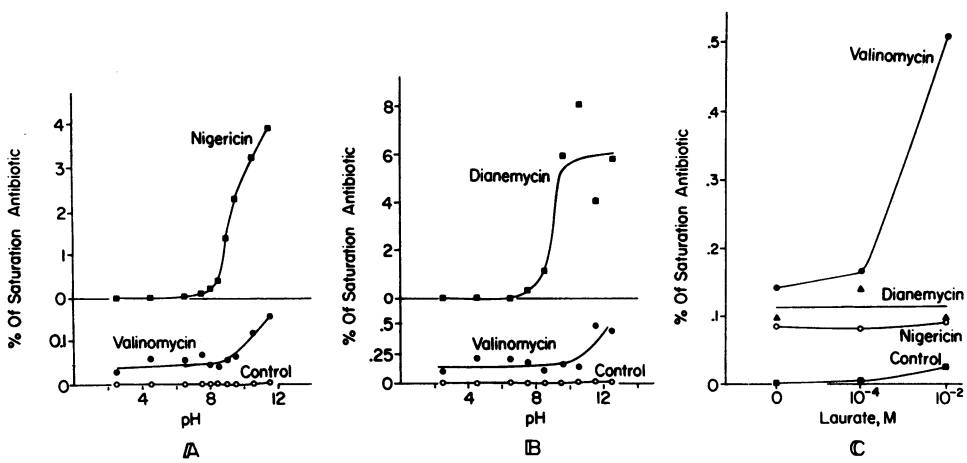

FIG. 4.—$^{86}Rb^+$-antibiotic complex formation. The migration of $^{86}Rb^+$ from aqueous buffer (0.5 ml, 10 mM glycine + 10 mM tricine adjusted to desired pH with HCl or $N(CH_3)_4OH$ into the nonaqueous phase (1 ml, 30% n-butanol-70% toulene) was measured by counting an aliquot of the latter phase by liquid scintillation with a PPO-POPOP-toluene-ethanol phosphor system. $^{86}Rb^+$ in the nonaqueous phase was expressed as the per cent saturation of the test antibiotic assuming 1:1 complex formation.

In *expt. A* the aqueous phase contained 33 μM RbCl, 5×10^6 cpm; the nonaqueous phase 210 μM nigericin or 140 μM valinomycin (^{86}Rb in antibiotic-free control negligible).

In *expt. B* the aqueous phase contained 93 μM RbCl, 4×10^6 cpm; the nonaqueous phase 75 μM dianemycin or 45 μM valinomycin.

In *expt. C* the aqueous phase contained 33 μM RbCl, 6.5×10^6 cpm, and the indicated concentrations of laurate, final pH, 6.5; the nonaqueous phase, 95 μM nigericin, 70 μM dianemycin, or 65 μM valinomycin.

The ionic preference of valinomycin complex formation is $Rb^+ > K^+ > Cs^+ \gg Na^+$, similar to that reported previously for the permeability sequence with valinomycin-treated artificial membranes.[8, 9] The biological significance of this ionic sequence with respect to the permeability of natural membranes has been discussed briefly.[9]

The valinomycin class of antibiotics induce electrically detectable effects on the model membrane systems,[8-10] either biionic potentials when different ion species and/or concentrations are opposed across the membrane, or the passage of current, i.e, lowering of membrane resistance, in an electric field. Both phenomena depend on the passage across the membrane of charged particles, either free cations or the charged ion-antibiotic complex.

It has been proposed that the valinomycin class antibiotics may create specifically tailored pores in lipid membranes through which ions may pass.[8, 25, 26] If the nigericin class antibiotics were to act similarly, electrogenic phenomena should also result. If, alternatively, the dissociable antibiotics carry ions across the membrane as paired-ion, neutral complexes as we propose, no translocation of charge and hence no electrically measurable effects would arise. Since natural membrane systems as well as bulk phase solvent partition systems exhibit nigericin-induced cation permeability, we presume that the artificial bimolecular leaflet system behaves similarly. No change in the ohmic resistance of a variety of membranes tested by Mueller and Rudin (personal communication) and ourselves was observed with either nigericin or dianemycin; hence, ions could traverse such membranes only as paired-ion complexes of no net charge. By analogy, it is likely that the members of the valinomycin antibiotic class also carry ions across lipid barriers as alkali complexes which in this case bear a net positive charge.

Since we propose that nigericin can function to transport protons, it is of interest that this property has also been suggested for the mechanism of action of uncoupling agents.[7, 27] The data of Figures 2, 3, and 4 are all consistent with this proposal. Other data, however, e.g., the H^+ flux in valinomycin-treated mitochondria,[4] and the pH dependency of the equilibrium between substrate systems and the endogenous pyridine nucleotides[28] suggest that mitochondria are intrinsically highly permeable to protons. Within this context we decided to reexamine the reports that oxidative phosphorylation could partially survive nigericin treatment. The conditions in Figure 5, i.e., high substrate concentration and mild hypotonicity, were chosen to facilitate substrate entry into K^+-depleted mitochondria. The ability of mitochondria to phosphorylate small pulses of adenosine 5′-diphosphate (ADP) is evidenced by the duration of both the respiratory burst and fluorescence decrease (i.e., pyridine nucleotide oxidation) as well as alkaliniza-

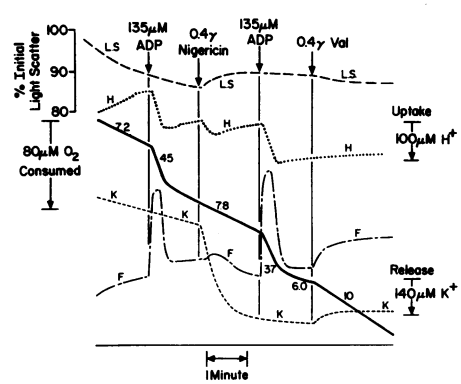

FIG. 5.—Effect of nigericin-induced K^+ discharge on oxidative phosphorylation. The system contained: 12 mM Tris glutamate; 12 mM malate; 10 mM Tris Cl; 5 mM KCl; 0.5 mM $MgCl_2$; 2 mM Tris PO_4; 120 mM sucrose; 3.6 mg protein/ml; pH 7.25, T = 22°. Trace identification as in Fig. 1

tion due to the reaction ADP + P_i + $\phi H^+ \rightarrow$ ATP, where ϕ is a function of pH.[20, 29] Since, by these criteria, phosphorylative capacity is scarcely affected following the release of endogenous K^+ by nigericin, the conclusion is supported that such inhibition as occurs is connected with the K^+ loss[11] which diminishes the accumulation of negatively charged substrate ions.[18, 30] Note that under these conditions of high substrate concentration, nigericin addition induces only a slight, transitory fluorescence decrease, and furthermore, in the absence of valinomycin, fails to stimulate respiration (cf. Fig. 1), indicating that K^+, in the form of a nigericin complex, is not available to the energy-linked mitochondrial ion pump.

Figure 5 is also highly relevant to the chemisosmotic hypothesis.[27] Here nigericin provides a sufficiently rapid ion bypass to discharge the mitochondrial K^+ and H^+ gradients, yet the metabolically produced energy persists in a form capable of driving oxidative phosphorylation at essentially the control rate.

Discussion.—In a series of papers Lardy and his co-workers have concluded that the nigericin-type antibiotics act by interfering with the availability of a high-energy intermediate to the mitochondrial ion pump.[11–14] We have here demonstrated, however, that the effects of nigericin are not restricted to the energized state of mitochondria, nor to mitochondrial systems. It has also been reported that the valinomycin-induced gross K^+ influx is not inhibited by dianemycin addition despite the rapid discharge of K^+.[21]

It is our conclusion that both the valinomycin and the nigericin classes of antibiotics induce alkali ion permeability in mitochondrial and other systems by carrying ions across lipid barriers as lipid-soluble complexes. Accordingly we propose to classify them generically as *ionophores* or *ionophorous agents.*

The ionophorous antibiotics are all of relatively low molecular weights, and exhibit an apparent complexing preference for K^+ and Na^+ ranging from as high as 10,000:1 for valinomycin in mitochondrial systems, to close to unity for the gramicidins[6] and dianemycin. They operate on a variety of natural and artificial lipid systems. Their alkali complexes presumably form and dissociate rapidly and are capable of rapid diffusion across lipid barriers (turnover numbers for valinomycin in mitochondria at room temperature are several hundred K^+/sec). Complexes of the valinomycin type have a net positive charge while those of the nigericin type exist as ion-pair complexes without net charge.

The major differences between the effects of the two classes of ionophorous agents on mitochondria could be accounted for if the nigericin group confers H^+ permeability and provides ion pathways across the mitochondrial membrane at random loci, while the valinomycin group interacts preferentially at the mitochondrial ion pump assembly (cf. refs. 16, and 31) without increasing H^+ permeability. Thus only the valinomycin class can promote the movements of alkali ions against a concentration gradient in mitochondria, while the nigericin class primarily facilitates the downhill dissipation of such concentration gradients.

The valinomycin-induced release of endogenous K^+ from mitochondria can be coupled to ATP synthesis but not the even more extensive K^+ release catalyzed by nigericin under identical conditions.[31] Reversal of ion uptake takes place when the nigericin-induced K^+ leak outpaces the valinomycin-induced, ion pump-assisted K^+ uptake. The nigericin-induced ATPase can also be explained by its effect on mitochondrial K^+ permeability. Despite the low affinity of nigericin for

the ion transport assembly, at higher concentrations it could deliver enough K^+ through the mitochondrial K^+ impermeable barrier locus, to the energy-dependent pump to facilitate an ATP-drive K^+ flux thereby inducing ATPase activity.

Impairment of related anion and cation fluxes as mitochondrial cations are released can produce secondary metabolic inhibitions which may obscure the primary effects of the antibiotics. Diminished translocation of components of the ATPase system, i.e., ADP, ATP, P_i (all anions presumably benefiting from the availability of cations for penetrating the mitochondrial membrane), could affect the nigericin-induced ATPase and may, in part, account for the inability to observe a dianemycin induced ATPase.[14]

Specific carrier substances for sulfate[32] and carbohydrate[33] have already been reported in bacteria. Because of their content of unnatural D configurations, ionophores such as valinomycin probably do not occur naturally in higher organisms, but nevertheless their properties support the plausibility of specific ion carriers of relatively simple structure and high turnover numbers. The existence of the ionophorous antibiotics therefore favors the likelihood of carrier mediated transport in biological systems (cf. ref. 34). Indeed, if the ion affinity center of an ionophore could be altered allosterically by energy-linked reversible configurational changes, it would acquire the requisites for promoting active transport.

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