

Potassium Ion-dependent Hydrolysis of Adenosine Triphosphate Induced by Nigericin in Mitochondria*

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SUMMARY

K^+ is required for the stimulation by the antibiotic nigericin of adenosine triphosphate hydrolysis in rat liver mitochondria. This action does not occur when K^+ is replaced by other monovalent cations. It requires slightly hypotonic conditions and is not related to the swelling phenomena linked to the accumulation of K^+ in mitochondria. ATPase is inhibited by small anion molecules to which the mitochondrial membrane is impermeable, less so if the anion is translocated inside the mitochondria. High concentrations of Rb^+ or Na^+ and low concentrations of Ca^{++} inhibit the ATPase stimulated by nigericin. The ATPase activity of submitochondrial sonic fragments, and of Racker's coupling factor F_1 , are not stimulated by nigericin.

β -Hydroxybutyrate and succinate partially inhibit the hydrolysis of ATP stimulated by nigericin in K^+ media. Neither glutamate, malate, pyruvate, nor α -ketoglutarate inhibits this enzymatic activity. The inhibition of ATPase by oxidizable substrates is ascribed to the reincorporation of the liberated inorganic orthophosphate into ADP by way of the phosphorylation linked to the oxidation of succinate and β -hydroxybutyrate.

Low concentrations of nigericin partially inhibit the ATPase stimulated by uncoupling agents requiring the presence of alkali metal cations for their mechanism of action; the inhibition depends on the ionic environment of the mitochondria. Nigericin does not affect ATPase activities induced by agents with no alkali metal cation requirement. Dianemycin, an antibiotic with properties similar to nigericin regarding induced ion movement and spectra of respiratory inhibition, does not stimulate the hydrolysis of ATP in any of several media tried.

The K^+ -dependent ATPase stimulated by nigericin appears not to be related to a translocation of alkali metal cations induced by the antibiotic. The possibility that the induction of this activity is related to the participation of a potassium-dependent step in the sequence of reactions leading to the synthesis of ATP in mitochondria is discussed.

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It has recently been shown that a group of compounds that specifically modify the vectorial movements of alkali metal ions in mitochondria also stimulate the hydrolysis of adenosine triphosphate with a strict dependency on the cation environment. Valinomycin (1) induces an increase in mitochondrial ATPase activity requiring K^+ , Rb^+ , or Cs^+ (2); commercial triamcinolone (3, 4) activates ATPase in the presence of K^+ or Rb^+ , less with Na^+ , and not with Li^+ or Cs^+ ; gramicidin (5) stimulates the hydrolysis of ATP in the presence of K^+ , Rb^+ , Cs^+ , Na^+ , or Li^+ ; the nonactin homologues (6) are active in the presence of Na^+ , K^+ , Rb^+ , or Cs^+ , and have little activity with Li^+ . A common feature of these compounds is their ability to promote the active uptake of alkali metal cations in mitochondria (2) supported by the high energy intermediates—collectively labeled $\sim W$ (7)—derived either from ATP or from the electron transfer chain.

The previous findings suggest that the alkali metal cation-dependent stimulation of ATP hydrolysis produced by these compounds occurs because of the energy demands imposed by the transport of ions. However, evidence will be presented in this paper to show that the activation of the alkali metal cation-dependent ATPase from mitochondria is not necessarily related to a predicted change in the pattern of ion movements induced in the mitochondrial membrane.

Recently we reported (8) some properties of the antibiotic nigericin involving specific respiratory inhibition coupled to the loss of alkali metal cations induced by this compound in mitochondria. On the basis of these observations, we suggested that the site of action of this antibiotic must be at the level of the ion-translocating mechanism of the mitochondrial membrane.

The present paper deals with the description of a unique mitochondrial ATPase activity induced, specifically in K^+ -containing media, by relatively high concentrations of nigericin. This effect on ATPase activity seems to be distinct from the action of nigericin in blocking the mitochondrial ion pump. It may be the result of the interaction of the antibiotic with a mitochondrial component that requires K^+ for its participation in the mechanism of oxidative phosphorylation.

EXPERIMENTAL PROCEDURE

Mitochondria were prepared from livers of male rats weighing 150 to 220 g according to the procedure described by Johnson and Lardy (9). The mitochondria were stored in 0.25 M sucrose at 0° and used within 1 hour of preparation.

The ATPase activity was measured by the method of Lardy and Wellman (10); specific conditions are described in the tables

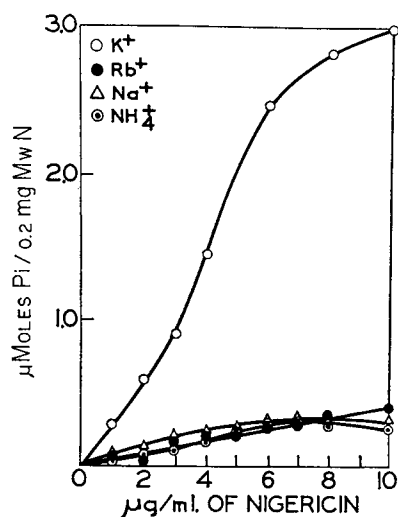


FIG. 1. The effect of the concentration of nigericin on the activity of mitochondrial ATPase in the presence of different alkali metal cations. The reaction mixture contained 6 mM Tris-ATP (pH 7.4), 10 mM triethanolamine (Cl^-) (pH 7.4), 50 mM alkali metal cation (Cl^-), and mitochondria from 0.06 g of liver in 0.3 ml of 0.25 M sucrose in a final volume of 1.0 ml at pH 7.4. Nigericin was added in 0.01 ml of a 25% solution of *N,N*-dimethylformamide in 96% ethanol. In this and the following figures, *Mw* on the ordinate denotes mitochondria washed with 0.25 M sucrose.

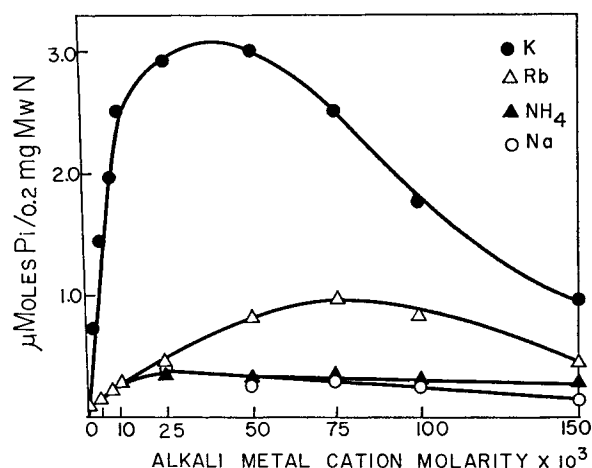


FIG. 2. The dependency on K^+ of the ATPase activity stimulated by nigericin. Experimental conditions were the same as in Fig. 1, except for the varying concentrations of the salt used to support the increase in enzyme activity induced by the antibiotic.

and figures. Inorganic phosphate was determined by the method of Sumner (11). The submitochondrial sonic fragments were prepared by the procedure described by Graven, Estrada-O., and Lardy (12). The coupling factor F_1 of Pullman *et al.* (13) was generously provided by Miss Chien Ho-Chiu.

Deionized water and highest purity chemicals were used throughout. The antibiotics used in this work were generous gifts from the following sources: monactin, Professor V. Prelog, Eidgenössischen Technischen Hochschule, Zurich; aurovertin, Drs. H. A. Nash and C. L. Baldwin, Pitman Moore Division, Dow Chemical Company; gramicidin B, Dr. Bernard Witkop, National Institutes of Health; valinomycin, Dr. J. C. McDonald, Saskatoon, Saskatchewan; 1,1,3-tricyano-2-amino-1-propene,

Dr. Floyd Eberts, The Upjohn Company; BA 180265, Dr. K. V. Rao, Pfizer Laboratories; and nigericin, Dr. R. Harned, Commercial Solvents Company. The Tris salt of ATP was obtained from Sigma Chemical Company.

RESULTS

Nigericin in concentrations from 1 to 10 μ g per ml induced the hydrolysis of ATP by mitochondria in media containing K^+ (Fig. 1). Maximal stimulation of ATP hydrolysis was induced by nigericin at a concentration of 10 μ g per ml; in other experiments, no further enhancement of this activation was observed at higher concentrations of the antibiotic. No significant activation of ATPase was detected when the concentration of nigericin in the medium was below 0.5 μ g per ml.

Alkali Metal Cation Requirement of ATPase—The induction of ATPase activity by nigericin was related to the concentration of K^+ in the medium (Fig. 2) with maximal activity between 25 and 50 mM. The apparent K_m for K^+ was 5 mM. KCl in concentrations above 50 mM inhibited the nigericin-stimulated ATPase, with complete inhibition at 300 mM. Although the potassium ion was responsible for part of the inhibition, as will be seen below, the anion (Cl^-) accompanying the monovalent metal cation affected the extent of this inhibition.

In the presence of Rb^+ , nigericin stimulated slightly the hydrolysis of ATP (Fig. 2). However, whereas K^+ has an apparent K_m of 5 mM, Rb^+ slightly stimulated the mitochondrial ATPase below 25 mM, and its activation was only 30% of that induced by nigericin in media containing K^+ . Na^+ or NH_4^+ , at concentrations in which K^+ (25 to 50 mM) produced a full activation of the enzyme, permitted only a minimal activation of ATPase (0.4 μ mole of P_i per 0.2 mg of mitochondrial nitrogen) (Figs. 1 and 2). Li^+ and Cs^+ did not support nigericin-induced ATPase activity at any concentration tested.

When the concentration of alkali metal cation was kept constant (75 mM), any replacement of K^+ with Na^+ resulted in a decrease in the ATPase induced by nigericin (Fig. 3). If NH_4^+ ,

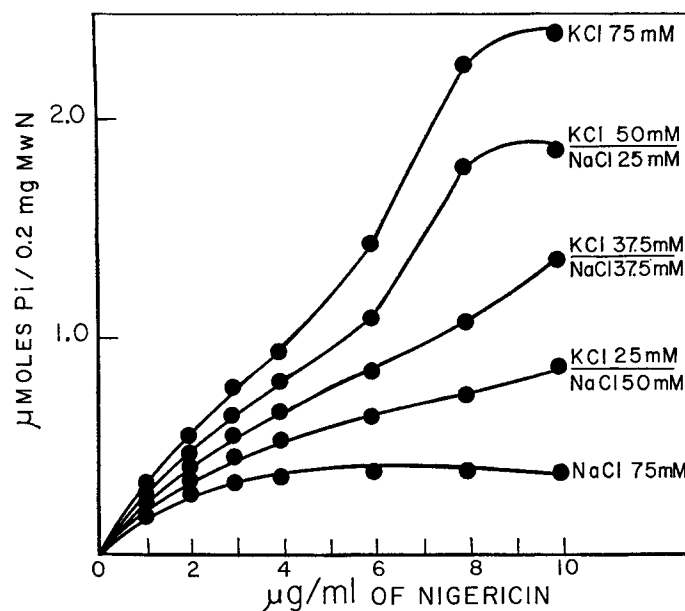


FIG. 3. The inability of Na^+ to replace K^+ on the activity of ATPase stimulated by nigericin. Experimental conditions were the same as in Fig. 1.

Li^+ , or Cs^+ was used in place of Na^+ , the results were similar to those presented in Fig. 3. Divalent metal ions did not support the induction of ATPase activity by nigericin.

pH Dependency of Nigericin-stimulated ATPase—In mitochondria incubated in a medium buffered with 8×10^{-3} M histidine, 10^{-2} M triethanolamine, and 20 mM acetate, nigericin induced an increase in ATPase activity with an apparent optimal peak at pH 6.3 and a progressive decrease of the enzyme activity as the medium approached pH 8.0 (●, Fig. 4). Subtracting the spontaneous, bell-shaped ATPase activity (○) from these values results in a curve with an optimal ATPase activity in-

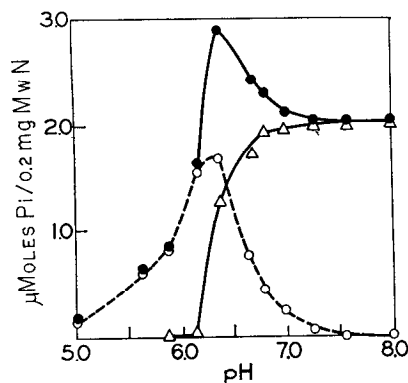


Fig. 4. The effect of pH on the K^+ -dependent ATPase from mitochondria stimulated by nigericin. The reaction system contained 6 mM Tris-ATP, 10 mM triethanolamine-HCl, 8 mM L-histidine, 20 mM acetate, and 50 mM KCl. The vessel contents were adjusted to the indicated pH values with HCl, and 10 μg of nigericin per ml were added to the corresponding media. Mitochondria containing 0.2 to 0.4 mg of nitrogen were added in 0.3 ml of 0.25 M sucrose. Final volume was 1.0 ml. ○—○, spontaneous ATPase induced by the pH change in K^+ media and hypotonic conditions; ●—●, activity of ATPase induced by nigericin at different pH values; △—△, corrected curve of the ATPase activity stimulated by nigericin obtained after subtracting the spontaneous ATPase from the ATPase in the presence of nigericin.

TABLE I

Effect of sucrose molarity on hydrolysis of ATP stimulated by nigericin in rat liver mitochondria

The reaction mixture contained 6 mM Tris-ATP, 50 mM KCl, 10 mM triethanolamine-HCl, mitochondria from 0.06 g of liver in 0.3 ml of 0.1 M sucrose, 10 μg of nigericin per ml, and sucrose to achieve the indicated concentrations in a total volume of 1.0 ml. Nigericin was added in a volume of 0.01 ml of a 25% solution of *N,N*-dimethylformamide in 96% ethanol. The mixture was incubated for 10 min at 30°. Average values of two experiments are presented. All values for P_i liberated have been corrected for ATP hydrolysis in corresponding control tubes without added nigericin.

Sucrose concentration	P_i liberated
mM	μmoles/0.2 mg N
30–60	2.50
80	2.25
105	1.69
130	1.56
180	0.30
230	0.20
280	0.03
300	0

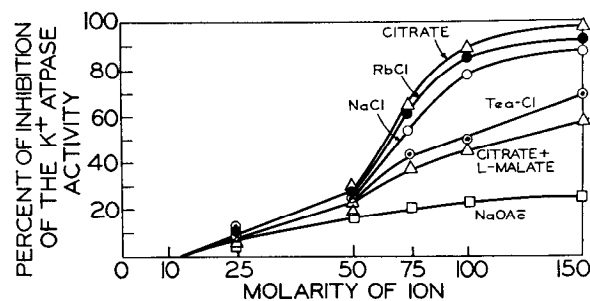


Fig. 5. The effect of anions Rb^+ and Na^+ on the K^+ -dependent hydrolysis of ATP induced by nigericin. The reaction system contained 6 mM Tris-ATP, 10 mM triethanolamine (Cl^-) (*Tea-Cl*), 50 mM K^+ acetate, mitochondria from 0.06 g of liver in 0.3 ml of 0.25 M sucrose, and the indicated additions in a final volume of 1.0 ml at pH 7.4. *NaOAc*, sodium acetate.

duced by nigericin at pH 7.0 and above (△). Nigericin induced no ATPase activity when the pH of the medium was less than 6.1.

Effect of Changes in Osmolarity on ATPase—Changes in the osmolarity of the media were found to have a marked effect on the ATPase induced by nigericin. When 50 mM KCl was present in the incubation medium, the addition of increasing amounts of sucrose (from 80 to 300 mM) progressively inhibited the ATPase activity induced by nigericin until none was detected at 300 mM sucrose (Table I). It appears that hypotonic conditions are required for the activation of ATPase by this antibiotic.

Effect of Anions on ATPase—High concentrations of potassium chloride inhibited the nigericin-activated ATPase as effectively as equivalent molarities of sucrose (Table I; Figs. 2 and 5). Since the mitochondrial membrane is relatively impermeable to Cl^- ions (14), the inhibition of ATPase was presumed to be a result of the change in osmolarity and not an inhibition caused specifically by Cl^- . To assess this possibility, the effect on nigericin-activated ATPase of increasing concentrations of different anions was studied (Table II). On the basis of the results, anions can be separated into two different groups: (a) those which inhibit the K^+ -dependent ATPase induced by nigericin (chloride, bromide, iodide, nitrate, and citrate) and (b) those which do not inhibit this ATPase activity (acetate, phosphate, and formate). Citrate at 50 mM inhibited ATPase 30% and at 150 mM inhibited completely. This inhibitory effect of citrate does not appear to be due to its chelating activity since EDTA at concentrations up to 60 mM did not inhibit the ATPase activity induced by nigericin. Bromide, nitrate, and iodide were less inhibitory than citrate, and chloride was even less effective.

As is shown in Table II, acetate, phosphate, and formate did not inhibit the ATPase activity appreciably (<30%, even at 150 mM). The anions which failed to inhibit the nigericin-induced ATPase are those to which the mitochondrial membrane is permeable (15). It would appear that the inhibitory effect of anions described above is not the result of simple change in the total osmolarity of the solution, but is related also to the permeability of the mitochondria to the anions. Support for this proposal comes from the results of the experiments in which L-malate (5 or 7.5 mM) was added to a medium containing increasing amounts of citrate (Table II and Fig. 5). L-Malate facilitates citrate penetration into mitochondria (16, 17). The presence of L-malate decreased the inhibition of nigericin-induced ATPase produced by citrate. Increasing the concentration of malate

TABLE II

Effect of various anions on K^+ -dependent ATPase of mitochondria stimulated by nigericin

Media contained 10 mM triethanolamine, 50 mM K^+ acetate, 6 mM Tris-ATP, mitochondria from 0.06 g of liver in 0.3 ml of 0.25 M sucrose, and the indicated additions, as K^+ salts, in a total volume of 1.0 ml with a final pH of 7.4. L-Malate, when present, was added in a concentration of 7.5 mM. Incubation was conducted for 10 min at 30°. The average values of duplicate experiments are given.

Anion	P _i liberated at anion concentrations of					
	0 mM	10 mM	50 mM	75 mM	100 mM	150 mM
	$\mu\text{moles}/0.2 \text{ mg N}$					
Bromide.....	3.0	3.0	2.0	1.0	0.75	0.48
Nitrate.....	3.0	3.0	0.65	0.48	0.25	0.04
Iodide.....	3.0	3.09	2.82	2.11	1.20	0.36
Chloride (Tris salt)....	2.5	2.42	1.90	1.65	1.25	0.85
Chloride (NH_4 salt)....	3.0	3.1	2.75	2.6	2.2	1.75
Citrate.....	2.5	2.28	1.12	0.45	0.06	0
Citrate + L-malate.....	2.5	2.28	1.65	1.35	1.05	0.85
Acetate.....	3.0	2.95	2.75	2.60	2.40	2.20
Formate.....	3.0	2.70	2.36	2.26	2.23	2.28
Phosphate ^a	3.0	3.0	3.0	2.9	2.7	2.4

^a In the presence of high phosphate, liberated P_i was determined by using an aliquot of a zero time sample as the blank in the spectrophotometer.

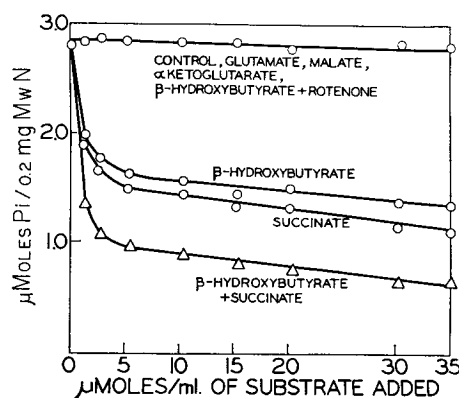


FIG. 6. The effect of substrate oxidation on the net formation of P_i in the presence of nigericin. Experimental conditions were the same as in Fig. 1. Mitochondria were incubated for 3 min at 30° with substrates before ATP and nigericin were added. Substrates neutralized to pH 7.4 with triethanolamine were used in this experiment. Rotenone (10^{-7} M), added in 0.01 ml of a 75% ethanol- H_2O solution, was used to inhibit β -hydroxybutyrate oxidation.

above 5 mM did not enhance the reversal of the inhibition produced by citrate. Malate alone had no effect (Fig. 6).

Inhibitory Effect of Rb^+ , Na^+ , and Ca^{++} on K^+ -dependent ATPase—The inhibition of nigericin-induced ATPase was greater with NaCl and RbCl than with triethanolamine chloride at comparable concentrations. It is very probable that this is the result of a specific inhibitory effect of Rb^+ and Na^+ on the K^+ requirement for the ATPase because neither lithium nor cesium chloride increased the inhibition above that observed with triethanolamine chloride. There was less inhibition with NH_4Cl than with triethanolamine chloride. The replacement of Cl^- by acetate decreased the inhibition produced by Na^+ (Fig. 5) as well as by higher concentrations of potassium ions.

Lardy, Johnson, and McMurray (18) reported that nigericin (2 μg per ml) induced a striking inhibition of the ATPase induced by 10^{-3} M CaCl_2 . As will be seen below, CaCl_2 also inhibited close to 70% of ATPase activity induced by nigericin when Ca^{++} was tested at concentrations of 2.5×10^{-3} M.

Inhibitory Effect of Substrates—Succinate and β -hydroxybutyrate at 5 mM inhibited the ATPase induced by nigericin 34 and 44%, respectively (Fig. 6). Further increases of succinate or β -hydroxybutyrate concentrations increased the inhibition slightly. However, the addition of both succinate and β -hydroxybutyrate enhanced the inhibition to 68%. Rotenone (10^{-6} M) reversed the inhibition of ATPase induced by 10 mM β -hydroxybutyrate (Fig. 6). None of the other substrates—glutamate, malate, pyruvate, or α -ketoglutarate—inhibited the ATPase activity at concentrations between 1 and 40 mM.

Effect of Nigericin on ATPase from Submitochondrial Preparations—Nigericin failed to enhance ATPase activity of sonic particles prepared from mitochondria or of the coupling factor F₁ of Pullman *et al.* (13) (Table III). Valinomycin and monactin also failed to stimulate the activity of ATPase in these preparations, one of which responds to 2,4-dinitrophenol. These findings indicate that the ATPase activity induced by nigericin, valinomycin, and monactin is dependent on the structural integrity of the mitochondria. Valinomycin and the nactins expend ATP by causing an increased transport of cations into, and diffusion from, mitochondria. It is possible that the high concentrations of nigericin also induce K^+ transport, but experimentally this has not been demonstrated because this antibiotic causes a rapid loss of accumulated ions from mitochondria (8). Dianemycin did not inhibit the K^+ -dependent ATPase induced by nigericin.

Inhibitory Effect of Nigericin on ATPase Stimulated by Different Compounds—The addition of nigericin (from 1 μg to 10 μg per ml) inhibited, to varying degrees, the ATPase induced by different agents in apparent correlation with the presence or absence of

TABLE III

Effect of nigericin and uncoupling agents on different ATPase preparations

The reaction mixtures contained 10 mM triethanolamine-HCl, 50 mM K^+ acetate, 6 mM Tris-ATP, 200 mM sucrose, and the indicated additions in a total volume of 1.0 ml; 10-min incubation at 30°. No sucrose was added for measuring the ATPase induced by nigericin in fresh mitochondria. MgSO_4 (10 mM) was added to the media for measuring ATPase from Racker's coupling factor F₁. The F₁ preparation had a specific activity of 43 when assayed according to Pullman *et al.* (13) with an ATP-regenerating system that removes inhibitory ADP.

Addition	P _i liberated			
	Fresh mitochondria	Submitochondrial sonic particles		Coupling factor F ₁
		No Mg^{++}	+1.5 $\times 10^{-2}$ M Mg^{++}	
	$\mu\text{moles}/0.2 \text{ mg N}$	$\mu\text{moles}/0.2 \text{ mg N}$		$\mu\text{moles}/\text{min}/\text{mg protein}$
None.....	0.24	0.19	2.96	19.6
Nigericin, 10 μg per ml...	3.56	0.07	1.85	20.4
Valinomycin, 10^{-7} M.....	3.70	0.14	2.13	19.1
Dianactin, 10^{-6} M.....	3.60	0.09	2.02	19.6
Gramicidin B, 10^{-6} M.....	3.52	0.06	2.85	19.7
2,4-Dinitrophenol, 10^{-6} M.	4.09	0.29	2.51	29.6

alkali metal cation requirements for the hydrolysis of ATP induced by those compounds.

Nigericin at a concentration of 2 μ g per ml inhibited the ATPase stimulated by valinomycin, nonactin, gramicidin D, and stendomycin A in K^+ media 46 to 67%; the action of the antibiotic BA 180265A was inhibited 31% (Table IV). As previously mentioned, the uncoupling of oxidative phosphorylation by valinomycin (2), gramicidin (5), the nonactin homologues (6), and stendomycin A (7), and the induction of ATPase by these agents as well as by BA 180265A (7), is dependent on the presence of alkali metal cations. Lardy *et al.* (18) also reported that nigericin at 2 μ g per ml inhibited 24% of the ATPase induced by

TABLE IV

Effect of nigericin on mitochondrial ATPase stimulated by different uncoupling agents

The reaction mixture contained 6 mM Tris-ATP, 37.5 mM KCl, 10 mM triethanolamine-HCl, 150 mM sucrose, mitochondria from 0.06 g of liver in 0.3 ml of 0.25 M sucrose, and the indicated additions in a final volume of 1.0 ml. Incubation was conducted for 10 min at 30°. Average values of duplicate experiments are presented.

Agent	P _i liberated		Inhibition
	Control	Nigericin, 2 μ g per ml	
	μ moles/0.2 mg N		%
None.....	0.23	0.62	
2-Amino-3,5-diiodobenzoate, 10 ⁻⁴ M.....	2.23	1.98	11
1,1,3-Tricyano-2-amino-1- propene, 5 $\times$ 10 ⁻⁴ M.....	1.60	1.56	
BA 180265A, 1 μ g.....	2.69	1.85	31
Monactin, 10 ⁻⁷ M.....	2.81	1.52	46
Valinomycin, 10 ⁻⁷ M.....	3.31	1.56	53
Gramicidin B, 10 ⁻⁶ M.....	3.52	1.51	57
Stendomycin A, 22.8 μ g.....	1.99	0.66	67

TABLE V

Effect of different uncoupling agents on ATPase stimulated by nigericin

The reaction mixture contained 6 mM ATP, 50 mM KCl, 25 mM sucrose, 10 mM triethanolamine buffer (pH 7.4), 10 μ g of nigericin, and mitochondria from 0.06 g of liver in 0.3 ml of 0.25 M sucrose in a total volume of 1 ml and was incubated for 10 min at 30°. Values represent the average of duplicate experiments which agreed closely.

Addition	P _i liberated		Inhibition
	μ moles/0.2 mg N		
None.....	3.06		
Valinomycin, 10 ⁻⁷ M.....	1.68		45
Dinaetin, 10 ⁻⁷ M.....	1.71		44
Gramicidin B, 10 ⁻⁶ M.....	1.77		42
CaCl ₂ , 2.5 $\times$ 10 ⁻³ M.....	0.97		68
MgCl ₂ , 2.5 $\times$ 10 ⁻³ M.....	2.83		7.5
1,1,3-Tricyano-2-amino-1-pro- pene, 5 $\times$ 10 ⁻⁴ M.....	3.63		0
2-Amino-3,5-diiodobenzoate, 10 ⁻⁴ M.....	2.94		3.9
Oligomycin, 2 μ g.....	0.18		94
Aurovertin, 2 μ g.....	3.02		0

10⁻⁴ M dinitrophenol, 75% of that induced by 100 μ g of deoxycholic acid per ml, and 90% of the enzyme induced by 10⁻³ M Ca⁺⁺. In this regard it is known that a portion of the dinitrophenol-stimulated ATP hydrolysis in liver mitochondria is also dependent on Na⁺ or K⁺ (10, 19) and the ATPase activity induced by deoxycholic acid is greater with K⁺ than with Na⁺ (4). Also, it is known that Ca⁺⁺ interacts in an apparent competition with the mechanism of K⁺ movements in mitochondria (20).¹

Nigericin (2 μ g per ml) did not inhibit the ATPase induced by 5 $\times$ 10⁻⁴ M 1,1,3-tricyano-2-amino-1-propene or 2-amino-3,5-diiodobenzoate (Table IV). The failure of nigericin to inhibit the ATP hydrolysis stimulated by triiodothyroacetate has been reported previously (18). No ion requirement is known for the hydrolysis of ATP induced by these last three compounds.

It was also found that the compounds with ability to induce hydrolysis of ATP that was partially inhibited by low concentrations of nigericin, inhibited the K⁺-dependent ATPase stimulated by higher concentrations of nigericin. Valinomycin, dinaetin, and gramicidin as well as Ca⁺⁺ inhibited the hydrolysis of ATP induced by nigericin 40 to 68%, while tricyanoaminopropene and 2-amino-3,5-diiodobenzoate were not inhibitory (Table V). Oligomycin completely inhibited the hydrolysis of ATP induced by nigericin, whereas aurovertin did not inhibit (Table V).

Differences between Nigericin and Dianemycin—Nigericin and dianemycin have been reported to be similar in their effect on the rate of oxidation of different substrates in mitochondria (18). It has also been observed that both antibiotics inhibit the ³²P_i-ATP exchange without uncoupling oxidative phosphorylation in mitochondria (18). Some differences between these antibiotics have suggested differences in their site of action (21). We have found recently that nigericin and dianemycin differ in their specificity for inducing the release of alkali metal cations (K⁺, Na⁺, and Li⁺) from mitochondria (12). In addition to these differences, dianemycin failed to induce ATPase in any of the media used. The mechanism by which nigericin and dianemycin inhibit the ³²P_i-ATP exchange reaction in mitochondria will be discussed elsewhere.

DISCUSSION

Nigericin is produced by a streptomycete isolated originally by Harned *et al.* from a Nigerian soil (22). It is a powerful inhibitor of the growth of a variety of microorganisms with the striking peculiarity that its lethal effect is reversed by the addition of K⁺ to the assay medium (23).

In the initial experiments with this antibiotic for studies of mitochondria (18), it was found to inhibit the oxidation of most of the DPN-linked substrates, but not of β -hydroxybutyrate and succinate. At low concentrations, nigericin did not uncouple oxidative phosphorylation with any oxidizable substrate but it inhibited the exchange of ³²P between inorganic phosphate and ATP. The same effects were produced in mitochondria by the antibiotic dianemycin.

In recent studies (8) it was found that both nigericin and dianemycin induced a rapid loss of alkali metal cations previously accumulated in mitochondria under the influence of valinomycin, monactin, or gramicidin B. The inhibition of the oxidation of glutamate and most other DPN-dependent substrates apparently

¹ S. N. Graven, S. Estrada-O., and H. A. Lardy, unpublished experiments.

TABLE VI

Effect of nigericin and dianemycin on hydrolysis of ATP stimulated by 10^{-6} M gramicidin B in Li^+ media

Media for assay of ATPase contained 6 mM Tris-ATP (pH 7.4), 10 mM triethanolamine-HCl, lithium chloride (adjusted to pH 7.4 with triethanolamine) at the indicated concentrations, sucrose to maintain the same isosmolarity in the different vessels, and 0.3 ml of a suspension of rat liver mitochondria in 0.25 M sucrose (equivalent to 0.06 g of liver, 0.3 to 0.4 mg of nitrogen). The experiment was conducted at 30° for 10 min. Average values of duplicates are presented.

Additions	P _i liberated at lithium chloride concentrations of			
	3.0 mM	15 mM	30 mM	60 mM
	μmoles/0.2 mg N			
None	0.41	0.32	0.26	0.25
Gramicidin B	1.18	1.79	1.57	1.49
Gramicidin + nigericin, 2 μg per ml	0.99	1.10	1.31	1.38
Gramicidin + dianemycin, 2 μg per ml	0.76	0.80	0.75	0.98

results from the necessity of a high salt concentration for these substrates, since it was reversed by 75 mM alkali metal cations. The oxidation of succinate, β -hydroxybutyrate, and, under some conditions, isocitrate appears to be less dependent on the ionic environment (8).

The present paper shows that nigericin induced a specific K^+ -dependent ATPase activity in mitochondria. The central question in these studies was whether this K^+ -dependent ATPase is (a) an essential part of the series of events which lead to the blocking of the uptake, or to the release of intramitochondrial alkali metal cations induced by nigericin, or (b) a collateral effect not directly related to the primary interaction of the antibiotic with the ion-translocating system of the mitochondrial membrane. The latter possibility appears to be the most likely based on the following considerations.

Optimal Concentration of Nigericin for ATPase and Ion Movements—Less than 0.3 μg of nigericin per ml (1 to 2 mg of mitochondrial nitrogen per ml) is sufficient to block the uptake of K^+ or Na^+ with the instantaneous inhibition of the oxidation of glutamate, malate, α -ketoglutarate, citrate, or pyruvate (8). However, no detectable ATPase activity is induced by nigericin below 0.5 μg per ml (0.2 to 0.3 mg of mitochondrial nitrogen per ml); 1 to 10 μg of nigericin per ml are required to induce ATPase (Fig. 1). Increasing the concentration of nigericin from 1 to 10 μg per ml did not change the pattern and efficiency of inhibition of respiration or of cation release from that induced by 0.3 μg per ml.

Optimal Osmolarity for Induction of ATPase Activity—The osmolarity required to demonstrate the induction of the K^+ -dependent ATPase is different from that required to demonstrate the effect of nigericin on ion movements and substrate oxidation in mitochondria. As seen in Table I, hypotonic conditions are required for the stimulation of the K^+ -dependent ATPase by nigericin. When nigericin was tested in an isotonic medium which was optimal for its effect on substrate oxidation and ion movements, it induced little ATPase activity.

Correlation between Ion Movements and ATPase Activity—As described in previous papers (8, 12) the addition of nigericin to

mitochondria prior to the addition of valinomycin and monactin prevents the uptake of K^+ induced by the latter compounds. Addition of nigericin after introduction of monactin or valinomycin in a medium containing K^+ , phosphate, Mg^{++} , and an oxidizable substrate causes the ejection of K^+ . The same pattern of responses is obtained with nigericin when gramicidin B is used to induce the uptake of Na^+ or Li^+ . However, no ATPase activity is induced by nigericin in the presence of Na^+ or Li^+ (Figs. 1 and 3). The K^+ specificity of the ATPase induced by nigericin does not appear to be related to the inhibition of alkali metal cation accumulation by liver mitochondria.

Effect of Inorganic Phosphate on ATPase and Alkali Metal Cation-dependent Respiration—We shall present data elsewhere showing that high concentrations of inorganic orthophosphate overcome the inhibitory effect of nigericin both on the active uptake of phosphate into mitochondria and on the oxidation of the substrates that have an alkali metal cation requirement for oxidation. However, high concentrations of inorganic phosphate do not inhibit the K^+ -dependent ATPase activity induced by nigericin (see Table II) as would be expected if the phosphate uptake and respiration coupled to the movement of monovalent cations, and the activity of ATPase, were directly related.

Differences between Nigericin and Dianemycin—Both nigericin and dianemycin induce the loss of intramitochondrial cations in a system having a rapid turnover of alkali metal cations (8, 12). However, dianemycin did not induce ATPase under a variety of conditions.

These several facts establish that the stimulation of K^+ -dependent ATPase and the changes in the movements of alkali metal cations linked to the respiratory activity of the mitochondria are independent but possibly related effects of nigericin. The partial inhibition by nigericin of the hydrolysis of ATP stimulated by valinomycin, the gramicidins, the nonactin homologues, etc., could be mediated by its interference with the incorporation of monovalent ions induced by these antibiotics in mitochondria. The ability of nigericin to induce the release of K^+ ions was greater than that of dianemycin when valinomycin or the nactins induced the accumulation of K^+ , while the ability of dianemycin to induce the release of Li^+ was greater than that of nigericin when gramicidin B was used to induce Li^+ accumulation in the mitochondria (12). In media containing Li^+ or Na^+ as the sole alkali metal cation, nigericin showed little capacity to inhibit the ATPase induced by gramicidin B, while dianemycin inhibited significantly (Table VI). Thus, nigericin and dianemycin follow a pattern of specificity for Li^+ or K^+ release similar to that found for their corresponding effects on ATPase stimulated by gramicidin, valinomycin, or the nactins in this paper.

The stimulation of the K^+ -dependent hydrolysis of ATP induced by nigericin may be the result of a specific interaction of the antibiotic with a mitochondrial component that requires K^+ for its participation in the mechanism of oxidative phosphorylation (24–26). Evidence bearing on this point will be published elsewhere.

The indication from Table I that nigericin induces ATPase only under hypotonic conditions requires revision in view of the fact that some electrolytes are not inhibitory. The effect of various anions on the K^+ -dependent ATPase induced by nigericin indicates the relation of this activity to the selective permeability properties of the mitochondrial membrane. Chloride, bromide, iodide and nitrate, when present at relatively high concentrations, inhibited the ATPase stimulated by nigericin (Ta-

ble I). Among these inhibitory anions are those demonstrated by Chappell and Crofts (14) and Chappell and Haarkoff (15) to be excluded from mitochondria. Conversely, phosphate, acetate, and formate, which are known to penetrate mitochondria, are far less inhibitory. Finally, citrate, which does not permeate (27, 28) and is inhibitory (Table I and Fig. 5), is rendered non-inhibitory by the addition of small quantities of L-malate, which permits citrate to penetrate mitochondria (16, 17, 29). That high concentrations of NH₄Cl only slightly inhibited the ATPase induced by nigericin (Table II) may be related to the observation that mitochondria take up NH₄⁺ with NH₃ being the probable penetrating species (5).

Thus the data of Chappell *et al.* (14, 15) obtained in swelling experiments and our observations on the activity of nigericin-induced ATPase in mitochondria lead to similar classifications of the various anions (Table VII). An association of ATPase activation with mitochondrial swelling has already been noted by Dianzani (30) in mitochondria suspended in hypotonic conditions. In certain cases ATPase activation may be accounted for by an altered permeability of the mitochondria which makes ATPase more accessible to its substrate (31). However, this does not seem to be simply the case for the K⁺-dependent ATPase stimulated by nigericin, because this antibiotic blocks the mitochondrial swelling (8, 12) induced in iso- or hypotonic solutions of KCl with substrate and acetate even in the presence of valinomycin or the nonactin homologues, which swell mitochondria when K⁺ is present (32, 33). It is of interest that mitochondria exposed to hypotonic conditions appear to maintain a specific permeability barrier to small anions. However, this same behavior of the membrane has been observed by others (34).

The apparent inhibition of nigericin-induced ATPase by succinate and β-hydroxybutyrate is characterized by a low substrate requirement, and by reversal by rotenone in the case of β-hydroxybutyrate. The effect can therefore be ascribed to the reincorporation of the liberated P_i into ATP by way of oxidative phosphorylation with these substrates but not with glutamate, malate, or α-ketoglutarate (8, 18). It has been suggested before by Chance and Ito (35) that the inhibition of endogenous ATPase of pigeon heart mitochondria induced by low concentrations of succinate could be mediated by a competition between the "hydrolysis" of an essential intermediate in ATPase activity and the binding of a component of this intermediate by DPNH formed in the energy-linked reduction.

If one assumes a single respiratory chain with different entry points for the substrates it is difficult to explain the finding that the combination of β-hydroxybutyrate and succinate produced substantially greater inhibition of the K⁺-dependent ATPase

activity than when either substrate was used alone. This has been noticed before by Wadkins and Lehninger (36) for the ATP-³²P_i exchange and the ATPase of mitochondrial subfragments sensitive to 2,4-dinitrophenol.

In conclusion, nigericin is an antibiotic which seems to have biphasic properties. At concentrations below 1 μg per ml it specifically interacts with the ion-translocating system of the mitochondrial membrane (8, 12) without induction of ATP hydrolysis. Above this concentration it induces an ATPase activity in mitochondria, adding its alkali metal cation-dependent uncoupling action (37) to its specific inhibitory effect on the mechanism of accumulation of K⁺ (8, 12) and inorganic phosphate (37, 38) in mitochondria.

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TABLE VII
Relative permeability of mitochondrial membrane to anions and correlation with anion effects on nigericin-induced ATPase

Nonpenetrating anions (inhibit K ⁺ ATPase)	Penetrating anions (slight or no inhibition of K ⁺ ATPase)	Permissive molecule
Chloride Bromide Iodide Nitrate Citrate	Acetate Formate Phosphate	L-Malate for citrate

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