

ALKALI METAL CATION RELEASE AND RESPIRATORY INHIBITION INDUCED BY NIGERICIN IN RAT LIVER MITOCHONDRIA*

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Nigericin is a monobasic acid antibiotic originally isolated by Harned *et al.*^{1, 2} The antibiotic analyzed for $C_{39}H_{69}O_{11}$ and the molecular weight of the sodium salt was found to be 736. Lardy *et al.*³ found nigericin and dianemycin to have the unusual property of inhibiting mitochondrial oxidation of glutamate and most other DPN-linked substrates and not inhibiting oxidation or phosphorylation with β -hydroxybutyrate or succinate. Nigericin and dianemycin were also observed to inhibit the exchange of P^{32} between inorganic orthophosphate and ATP. These antibiotics did not uncouple oxidative phosphorylation.³ In recent studies, nigericin⁴ and dianemycin were found to inhibit the uptake of K^+ induced by monactin or valinomycin in mitochondria. Nigericin and dianemycin cause a rapid loss of alkali metal cations which have accumulated in mitochondria under the influence of a variety of agents. The following is a report of the effect of nigericin on alkali metal cation movements and substrate oxidation in rat liver mitochondria. Experiments with this antibiotic have disclosed an unexpected difference between two groups of substrates with respect to alkali metal cation requirement for oxidation.

Experimental Materials and Methods.—Preparation of mitochondria: Mitochondria were obtained from the liver of male, white rats of the Sprague-Dawley strain by standard procedures,⁵ except that the homogenization medium contained 0.25 *M* mannitol, 0.08 *M* sucrose, and 0.01 *M* EDTA. The mitochondria were washed 3 times and resuspended to a concentration of 1 ml per gm of original liver in a medium of 0.25 *M* mannitol and 0.08 *M* sucrose. The experiments were begun as soon as the mitochondria were prepared, and completed within 4 hr.

The experiments were performed using an apparatus which simultaneously monitors and records concentrations of O_2 , K^+ , or Na^+ , and H^+ , light scatter, and fluorescence.^{6, 7} It was designed, developed, and constructed by B. Chance, D. Mayer, and B. Pressman at the Johnson Foundation, University of Pennsylvania. Light (520 $m\mu$) scatter was monitored at 180° . The concentrations of K^+ and Na^+ were monitored with Beckman cation-sensitive electrodes #39046 and 39047. The concentration of H^+ was monitored with a Beckman 39030 combination pH electrode. Oxygen was monitored with a collodion-covered, vibrating platinum electrode. After suitable amplification the data were recorded using a 6-channel Honeywell 1508 Visicorder. The cuvette was maintained at 28° with a constant temperature jacket. The cuvette volume was 5 ml.

Reagents: The Tris salt of ATP was obtained from the Sigma Co. The antibiotics used were obtained from the following sources: monactin—Prof. V. Prelog, Zurich; gramicidin B—Dr. Bernard Witkop, National Institutes of Health; antimycin A—Dr. T. Kagawa, Kyowa Fermentation Ind. Co.; nigericin—Dr. R. Harned, Commercial Solvents Corporation; and valinomycin—Dr. J. C. McDonald, Saskatoon, Saskatchewan.

Results.—The introduction of monactin or valinomycin into a medium containing mitochondria, K^+ , Mg^{++} , PO_4 , or acetate and an oxidizable substrate induces the

uptake of K^+ by the mitochondria, stimulates oxygen uptake, and causes the mitochondria to swell^{4, 7-10} (Fig. 1). The subsequent addition of nigericin causes ejection of K^+ , contraction of the mitochondria, and inhibition of respiration with L-malate as the substrate. The addition of citrate restores respiration (in the presence of L-malate) but does not induce K^+ uptake (not shown on figure). The introduction of nigericin prior to the addition of monactin or valinomycin prevents the uptake of K^+ or the stimulation of respiration when L-malate, glutamate, α -ketoglutarate, or pyruvate (with or without L-malate) are the substrates. When succinate or β -hydroxybutyrate are the oxidizable substrate, nigericin induces release of alkali metal cation and contraction of the mitochondria but does not inhibit respiration (Fig. 2). If NaCl replaces KCl in the medium (Fig. 2), Na^+ uptake is induced by gramicidin B.^{7, 10} The subsequent addition of nigericin induces the release of Na^+ and contraction of the mitochondria. Addition of nigericin prior to the addition of gramicidin B blocks the uptake of Na^+ by the mitochondria. If LiCl replaces NaCl in the medium, gramicidin B will induce the uptake of Li.¹⁰ The addition of nigericin induces the release of Li^+ from the mitochondria but at a slower rate than for Na^+ or K^+ .

Loss of intramitochondrial K^+ induced by nigericin appears to inhibit respiration with some substrates, e.g., L-malate, glutamate, α -ketoglutarate, or pyruvate, but does not inhibit respiration with succinate or β -hydroxybutyrate. In the presence of citrate, isocitrate, or *cis* aconitate, oxygen uptake induced by monactin or valinomycin is inhibited by nigericin in the absence of L-malate (Fig. 3). The relation between L-malate and the entry of these substrates into mitochondria has been reported by Chappell¹¹ and Ferguson and Williams.¹² Respiratory stimulation, K^+ uptake, H^+ ejection, and mitochondrial swelling are induced by monactin and reversed by nigericin (A in Fig. 3). Subsequent additions of isocitrate and D-malate were without effect, but the addition of 24 μ moles of L-malate stimulates respiration. Respiration with citrate (or isocitrate) and L-malate as substrates is partially inhibited by nigericin, an effect midway between the nearly total inhibition with L-malate, glutamate, α -ketoglutarate, or pyruvate and the lack of inhibition with succinate and β -hydroxybutyrate.

To determine the role of alkali metal cation in the inhibition of glutamate oxidation induced by nigericin, a series of experiments were performed with varying concentrations of NaCl. The results with a few representative concentrations are shown in Figure 4. Nigericin inhibited glutamate oxidation when the concentration of NaCl was below 30 mM. The effect of nigericin was partially reversed at

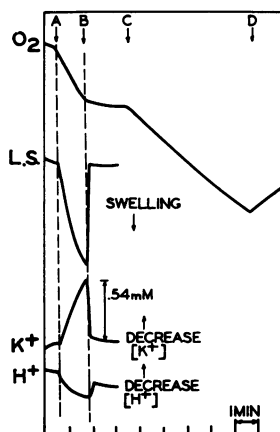


FIG. 1.—Effect of monactin and nigericin on the rate of oxygen consumption, light scattering, and the transport of K^+ and H^+ by liver mitochondria. A downward deflection of the light scattering trace (L.S.) is associated with swelling of the mitochondria. An upward deflection in the K^+ or H^+ tracing represents a decrease in the concentration of these ions in the medium or an uptake of K^+ or H^+ by the mitochondria. The medium contained 15 mM KCl, 8 mM Tris PO_4 , 3 mM $MgCl_2$, 0.16 M sucrose, 12 mM L-malate, and mitochondria equivalent to approximately 1.2 mg nitrogen in 5 ml vol at 28°C. Additions were as follows: A, monactin 1.5×10^{-3} μ moles; B, nigericin 2.8×10^{-3} μ moles; C, citrate (TEA neutralized) 24 μ moles; and D, antimycin A, 10 μ g.

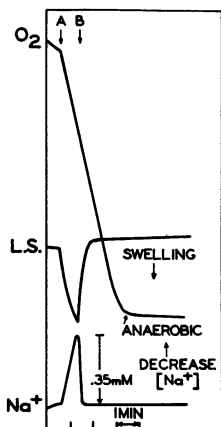


FIG. 2.—Effect of gramicidin B and nigericin on the rate of oxygen consumption, light scattering, and transport of Na^+ by liver mitochondria. The tracings are explained in the legend to Fig. 1. The medium contained 6 mM NaCl, 8 mM Tris PO_4 , 3 mM MgCl_2 , 0.16 M sucrose, 12 mM succinate, and mitochondria equivalent to 1.2 mg nitrogen in 5 ml vol at 28°C . The additions were as follows: A, gramicidin B 2×10^{-3} μmoles ; and B, nigerin 2.8×10^{-3} μmoles .

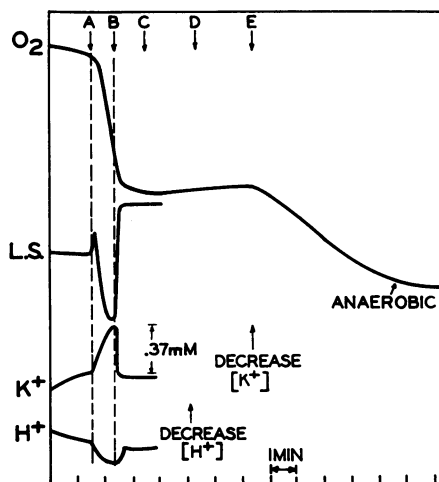


FIG. 3.—Effect of monactin, nigericin, D- and L-malate on the rate of citrate oxidation, light scattering, and the transport of K^+ and H^+ by rat liver mitochondria. The tracings are as in Fig. 1. The medium contained 15 mM KCl, 8 mM Tris acetate, 3 mM MgCl_2 , 0.16 M sucrose, 12 mM citrate, and mitochondria equivalent to 1.2 mg nitrogen. The additions were as follows: A, monactin, 1.5×10^{-3} μmoles ; B, nigericin 2.8×10^{-3} μmoles ; C, isocitrate 24 μmoles ; D, D-malate 15 μmoles ; and E, L-malate 24 μmoles .

NaCl concentration between 30 and 75 mM and nearly totally reversed at Na^+ concentrations above 75 mM. Similar previously reported⁴ experiments using KCl as the monovalent cation and monactin as the inducer of respiration gave nearly identical results. The respiratory inhibition induced by nigericin with glutamate, L-malate, α -ketoglutarate, or pyruvate as substrate can be prevented by concentrations of alkali metal cation above 75 mM.

The effect of nigericin on respiration induced by monactin and valinomycin in high and low K^+ concentrations was also observed in Warburg manometric experiments over a more extended period of time. Results from a typical experiment with glutamate as substrate are presented in Table 1. With a low concentration of K^+ (1 mM) in the medium, nigericin inhibited respiration 80 to 90 per cent. Often

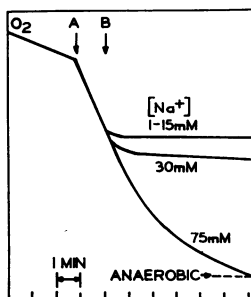


FIG. 4.—Effect of gramicidin B and nigericin on the rate of oxygen consumption in media of different NaCl concentrations. The medium contained NaCl as indicated in the figure, 8 mM Tris PO_4 , 3 mM MgCl_2 , 0.16 M sucrose, 12 mM glutamate, and mitochondria equivalent to 1.2 mg of nitrogen. The additions were as follows: A, gramicidin B 1.5×10^{-3} μmoles ; and B, nigericin 2.8×10^{-3} μmoles . The resting rates of O_2 consumption were 6.5–12.5 $\mu\text{moles O}_2/\text{gm protein}/\text{min}$. The rates of O_2 consumption induced by gramicidin B were 39–50 $\mu\text{moles O}_2/\text{gm protein}/\text{min}$.

TABLE 1
EFFECT OF HIGH AND LOW K^+ CONCENTRATION ON RESPIRATORY INHIBITION
PRODUCED BY NIGERICIN

Antibiotic	1.0 mM K^+ QO ₂ N	100 mM K^+ QO ₂ N
Valinomycin	208	385
Valinomycin + nigericin	23	126
Monactin	163	211
Monactin + nigericin	24	292

The medium contained 3 mM MgCl₂, 10 mM Tris PO₄, 10 mM glutamate, 40 mM sucrose at 100 mM K^+ , and 200 mM at 1 mM K^+ . Final volume = 3 ml; pH, 7.4 and T , 30°C. When present, monactin and valinomycin were at 5×10^{-7} M and nigericin was 2×10^{-4} M.

at high concentrations of K^+ , nigericin increases the rate of respiration above that induced by monactin as occurred in the experiment shown in the table.

Discussion.—Lardy *et al.*³ reported a 90 per cent inhibition of glutamate oxidation by nigericin at an antibiotic concentration of 1 μ g/3 ml in a K^+ -containing medium. The same concentration of nigericin produced only a 25–30 per cent inhibition of succinate or β -hydroxybutyrate oxidation. Nigericin did not uncouple oxidative phosphorylation but did inhibit the ATP- P_i exchange. Nigericin was also found to inhibit the ATPase induced by Ca^{++} and deoxycholate but not the ATPase activity induced by dinitrophenol or triac.³ In recent studies by Estrada *et al.*,¹³ nigericin at higher concentrations induced ATPase activity in a medium containing K^+ . The ATPase-inducing activity was specific for a K^+ -containing medium. This ATPase-inducing activity of nigericin is difficult to reconcile with its lack of specificity for release of alkali metal cation.

Sallis, DeLuca, and Martin¹⁴ recently reported an inhibition by nigericin of parathyroid hormone-dependent uptake of P_i supported by malate + pyruvate or α -ketoglutarate. The inhibition was not observed if succinate was the substrate.

From the data presented, it is proposed that nigericin induces the loss of alkali metal cation from mitochondria or prevents its uptake in a system in which there is a rapid turnover of cations. As noted, the releasing activity lacks the cation specificity observed for the ATPase activity of the antibiotic.

Since the inhibition of glutamate, α -ketoglutarate, L-malate, and pyruvate can be reversed by a high concentration of alkali metal cation in the medium, it would appear that these substrates require a relatively high concentration of alkali metal cation for oxidation with or without phosphorylation. The oxidation of succinate and β -hydroxybutyrate appears to proceed in the presence of much lower alkali metal cation concentrations. These observations might be reconciled by proposing separate compartments in mitochondria for the oxidation of the two classes of substrates. The effect of nigericin on substrate incorporation, ATP- P_i exchange, induction of ATPase, and the discussion of the compartmentation concept will be presented in subsequent reports.^{10, 13}

Since nigericin induces the release of alkali metal cation without inhibiting respiration or phosphorylation with succinate or β -hydroxybutyrate as substrate, the site of action must be at the level of the ion translocating mechanism in the membrane. The effects on substrate oxidation are apparently secondary to the loss of cation. This mode of action is analogous to the action of the polyene antibiotics on fungi where certain glycolytic enzymes are inhibited as a result of the K^+ loss from the cells.^{15–17}

Summary.—The antibiotic nigericin blocks the uptake of alkali metal cations induced in rat liver mitochondria by gramicidin, monactin, or valinomycin. It also causes rapid release of previously accumulated cations. In media of low to moderate alkali metal concentration, nigericin inhibits the oxidation of pyruvate, glutamate, α -ketoglutarate, and malate but not of succinate or β -hydroxybutyrate, nor of isocitrate in the presence of L-malate. The inhibition of substrate oxidation by nigericin can be overcome by high concentrations (*ca.* 75 mM) of alkali metal cations in the medium. The high internal alkali metal cation concentration required for mitochondrial oxidation of the first group of substrates can be achieved by concentration from a low cation medium. In the presence of nigericin, which blocks the cation pump, the requirement is met only by a high external concentration of cation.

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