EFFECT OF ENDOTOXINS OF Vibrio cholerae ON RESPIRATION OF LIVER MITOCHONDRIA IN THE PRESENCE OF KREBS' CYCLE SUBSTRATES

M. N. Dzhaparidze, L. A. Martens, and V. D. Egorova

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Endotoxins in Vibrio cholerae inhibit respiration of the liver mitochondria of rats and guinea pigs in the presence of malate, oxaloacetate, α-ketoglutarate, and pyruvate. The toxins did not affect oxidation of succinate, urate, and reduced NAD.

Vibrio cholerae has several types of toxins but their role in the pathogenesis of cholera has not yet been fully explained [5, 8].

The object of this investigation was to study the inhibitory effect of cholera endotoxin on the process of hydrogen transfer in the respiratory chain of the mitochondria in experimental animals, using intermediates of the Krebs' cycle as respiration substrates.

EXPERIMENTAL

Endotoxins of V. cholerae were prepared by the methods of Boiven and Watanabe [10], and isolated by filtration through Sephadex G-200 [4]. The molecular weight of the endotoxins, determined by fractionation on a column with Biogel R-300, was about 300,000.

TABLE 1. Effect of Cholera Endotoxin on Intensity of Respiration in Liver Mitochondria of Guinea Pigs (M ±m; n = 5-10)

Substrate	Absorption of oxygen (in μmoles/min/100 mg protein)			
	normal		toxin added ($8.4 \cdot 10^{-6}$ mM, after Boiven)	
	intact mitochondria	mitochondria treated with hypotonic solution	intact mitochondria	mitochondria treated with hypotonic solution
α-Ketoglutarate	17,0±1,63	24,5±2,03	1,0±0,06	1,5±0,08
" + Malate	15,2±1,00	21,2±1,80	1,0±0,06	1,5±0,07
" + NAD·H (+ H^+)	—	27,6±1,90	—	7,8±0,48
" + Cholera O-antiserum	—	22,8±2,63	—	21,3±2,32
" + Normal "	—	26,6±2,58	—	2,0±0,14
Malate	19,5±1,55	22,0±1,99	1,4±0,12	1,6±0,10
" + NAD·H (+ H^+)	—	30,2±2,24	—	16,0±1,20
" + Cholera O-antiserum	—	24,3±1,96	—	22,1±1,87
Oxaloacetate	23,0±1,66	25,5±1,94	6,0±0,72	7,2±0,64
Pyruvate	21,8±2,00	28,4±1,80	6,4±0,82	4,5±0,76
" + Malate	42,4+3,10	—	12,8±1,00	—
" + NAD·H (+ H^+)	—	38,0±3,20	—	10,8±0,62
Citrate	7,5±0,65	10,2±1,42	7,8±0,91	10,8±1,00
Succinate	28,3±2,46	38,2±2,82	26,2±1,96	34,3±3,15
NAD·H (+ H^+)	—	7,2±0,62	—	8,0±0,74

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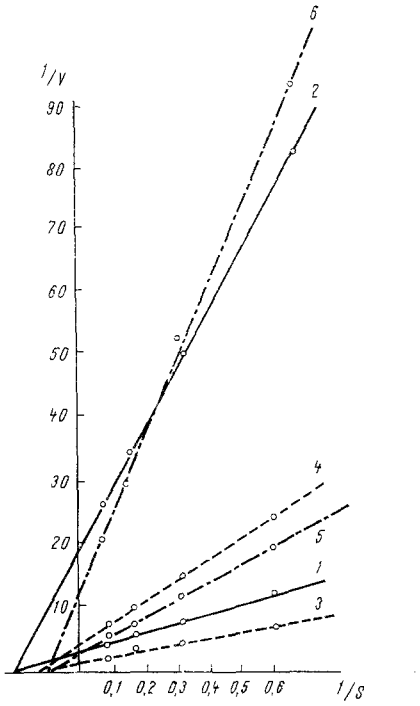

Fig. 1. Noncompetitive character of inhibition of mitochondrial respiration in albino rat liver by cholera endotoxin (4.2×10^{-6} mM). Reaction in the presence of: 1) malate; 2) malate + toxin; 3) α-ketoglutarate; 4) ditto + toxin; 5) pyruvate; 6) ditto + toxin. Ordinate: reciprocal of substrate concentration (in mM); abscissa: reciprocal of reaction velocity (see Table 1).

Albino rats and guinea pigs were used as experimental animals. Tests were carried out on mitochondria isolated from the liver by differential centrifugation of a homogenate prepared in isotonic sucrose solution (0.25 M with 0.001 M EDTA [3]). The mitochondria were broken up with hypotonic sucrose solution [3].

For determination of the oxygen absorption by a manometric method in a Warburg apparatus the experimental mixture contained (in moles) K_2HPO_4 0.02, $MgCl_2$ 0.01, KCl 0.2, ATP 0.0029, and 0.05 M tris-buffer (pH 7.4), 3 ml of each. The respiration substrates (pyruvate + malate, succinate, malate, α-ketoglutarate, oxaloacetate, and NAD · H_2) were added to the samples in an amount of 20 μmoles, unless mentioned specifically to the contrary in the text. The reaction mixture was incubated in air at 28°. The results were read every 10-20 min and expressed as μmoles O_2 utilized per minute, and calculated per 100 mg mitochondrial protein (protein was determined by Lowry's method [9]).

Besides the oxygen absorption, the decrease in concentration of the original respiration substrate also was determined. The content of the acids was determined by paper chromatography [1, 7].

EXPERIMENTAL RESULTS

The results given in Table 1 show that the endotoxin sharply inhibited respiration of the mitochondria when malate, α-ketoglutarate, oxaloacetate, and pyruvate were used as substrates, but in the presence of succinate and citrate, the absorption of oxygen was unchanged by endotoxins. This action of the endotoxin was unconnected with changes in permeability of the mitochondrial membranes. In the case of mitochondria treated with hypotonic sucrose solution, permeability to exogenous substrates is increased [3]. However, the percentage inhibition of respiration in that case was indistinguishable from that observed in the experiments with intact mitochondria.

Parallel determinations of the decrease in concentration of respiration substrates (malate, α-ketoglutarate, oxaloacetate, pyruvate) showed that this corresponded completely to the quantity of oxygen absorbed.

All the above remarks apply equally to the action of preparation of endotoxins obtained by Watanabe's method and isolated on a column with Sephadex G-200. No difference was found between the actions of preparations from strains of the Ogawa or Inaba types of the classical cholera vibrio or of the El Tor vibrio.

The endotoxin of V. cholera thus inhibits oxidation of malate, pyruvate, and α-ketoglutarate but does not affect the conversion of succinate and citrate. It is evident that those components of the electron transfer system which are common to these substrates cannot have been blocked by the toxins. Since oxidation of succinate is not inhibited by endotoxin, the transfer of hydrogen along the respiratory chain was disturbed, not in the region of Hatefi's complex I, but in the region of complex II [2]. The dehydrogenase (1.699.3) included in this particular complex is known to oxidize reduced NAD. This substrate was therefore tested in order to determine the site of action of cholera endotoxin on the mitochondrial respiratory chain. The mitochondria were treated with hypotonic solution in order to render the membranes permeable to reduced NAD. Absorption of oxygen in the presence of reduced NAD was not inhibited by the toxin (Table 1). These observations suggested that the direct site of action of endotoxin is the dehydrogenase connected with NAD. It must be noted that the toxin did not act in an identical manner on dehydrogenases.

The inhibitory effect of the toxin on oxidation of malate was diminished by about 75% in the presence of reduced NAD. Reduced NAD did not exhibit this positive effect on pyruvate- and α-ketoglutarate-dehydrogenase systems. In these experiments the oxygen consumption was probably due to oxidation of NAD · H

(+ H^+) only. This problem of the abolition of inhibition will be studied in more detail later. So far as the absence of inhibition of respiration when citrate was used as substrate is concerned, it is known that the NADP-linked isocitrate-dehydrogenase (1.1.1.42) is the most active of these enzymes in the tissues. In all probability these enzyme systems are insensitive to the action of cholera endotoxin.

Having determined the probable site of action of cholera endotoxin on the mitochondrial respiratory chain, the next step was to study some kinetic characteristics of inhibition.

Inhibition of mitochondrial respiration took place rapidly in the presence of malate, pyruvate, and α-ketoglutarate, and when a manometric method of investigation was used no change in the intensity of inhibition was observed with time. Endotoxin obtained by Boivin's method inhibited respiration under the above-mentioned experimental conditions by 30-40%, even in concentrations of the order of 10^{-6} mM, and in the course of 1.5 h of observation the percentage of inhibition was not increased. Since relationships between inhibitor and enzyme of this type are characteristic of reciprocal inhibition, an attempt was made to restore the activity of the system by removal of the inhibitor. For this purpose, cholera O-antiserum was used. This antiserum was added to the Warburg vessels 30-40 min after the beginning of inhibition of respiration by the toxin. As a first step, the doses of antiserum neutralizing the endotoxin in the tubes were chosen. Completeness of neutralization was verified by the gel-diffusion reaction.

The results given in Table 1 show that addition of the specific O-antiserum restored mitochondrial respiration in the presence of α-ketoglutarate and malate.

Having established the reversible character of the inhibition, an attempt was next made to determine the type of inhibition of NAD-dependent dehydrogenases by endotoxin.

Kinetic curves plotted by the Lineweaver–Burk method [6] are given in Fig. 1. All the experiments were carried out with mitochondria treated with hypotonic sucrose solution. Analysis of the curves shows that inhibition of these dehydrogenases by cholera endotoxin is noncompetitive in character, at least relative to the substrate.

Since one of the main causes of the toxic action of V. cholerae is considered to be the inhibition of active sodium transport through the membrane, it is natural to suggest that inhibition of respiration in the mitochondria discovered in these experiments and inhibition of the NAD-dependent dehydrogenases in the mitochondria play an important role in the pathogenesis of cholera.

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