ACTION OF PLAGUE MURINE TOXIN ON MAMMALIAN MITOCHONDRIAL RESPIRATION

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As an approach to the study of the mechanism of action of the murine toxin of *Pasteurella pestis*, experiments have been undertaken to determine its effect on mitochondria isolated from species sensitive and resistant to the action of the toxin. Studies to date have shown the toxin to be a relatively pure proteinaceous material by physical, chemical (Ajl *et al.*, 1955), and immunological analysis (Ajl *et al.*, 1958; Spivack and Karler, 1958). Ajl (1955) and co-workers reported the inhibition of keto acid oxidation by the toxin in cell-free extracts of *Escherichia coli* and crude mouse liver homogenates. These findings suggested an investigation of the effect of the toxin at the active site of respiration in animal tissues.

It has been found that an apparent association exists between the ability of plague murine toxin to inhibit the respiration of mitochondria from certain species and susceptibility of those animal species to the action of the toxin. Whereas plague toxin inhibits the respiration of heart mitochondria obtained from the toxin susceptible mouse and rat, it has little or no effect on those from the toxin refractory rabbit.

MATERIALS AND METHODS

Mammalian heart mitochondria were prepared and assayed for oxidation and oxidative phosphorylation as described elsewhere (Packer, 1957). Brain mitochondria were prepared according to Brody and Bain (1952). A sensitive polarographic method was employed for the measurement of oxygen consumption. A platinum-silver electrode couple polarized at 0.6 volts as recommended by Davies and Brink (1942) was employed. The current from the electrodes was measured and recorded with a General Electric model CE-5 photoelectric potentiometer. The reaction mixture (1.0 ml) was contained in a 5-ml test tube into which the electrodes were inserted and which was rotated at 60 rpm by a synchronous motor. The current generated at the electrodes was directly proportional to the dissolved oxygen concentration. Thus, as oxygen was consumed, a decrease in current occurred indicated by a downward deflection of the recorded curve. From the slope of the time curve, the rate of oxygen consumption in moles per liter per second was calculated. The electrode was calibrated with air saturated medium. At 26 C, the solubility of oxygen was taken as 240 μmoles per L. Polarographic measurements of oxidative phosphorylation were also carried out in this instrument as described by Chance and Williams (1955).

Plague toxin was prepared from an avirulent strain of *P. pestis*, "Tjiwidej," as reported earlier (Ajl *et al.*, 1955). Preparations with an LD_{50} of less than 1 μg for 16 to 18-g mice were used in these experiments. This material was equally toxic on a weight basis (56 μg/kg) to infant and adult mice but was not lethal to 200-g rabbits in concentrations up to 10 mg when injected intraperitoneally.

Inactive toxin was prepared by heating a dilute solution of the material at 100 C for 45 min and was not lethal in 5-mg amounts when administered intraperitoneally in mice.

The Vi antigen of *E. coli* and the O antigen of *Salmonella typhosa* were prepared by the method of Webster and associates (Webster *et al.*, 1952; 1955) and kindly supplied by Dr. A. Mandel and C. E. Buckler.

RESULTS

Initial experiments were carried out with rat heart mitochondria (sarcosomes) since this animal, like the mouse, is highly susceptible to murine plague toxin. The effect of 0.5 mg of the toxin in 1 ml reaction mixture during respiration

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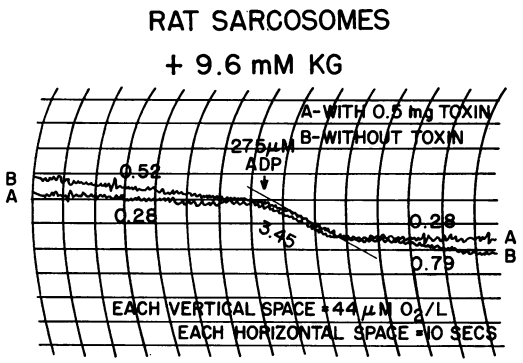

Figure 1. Effect of plague toxin on oxidative phosphorylation during α-ketoglutarate (KG) respiration in rat heart sarcosomes. The reaction mixture in the cuvette (1.0 ml) contained 1.69 mg sarcosomal protein, 0.32 M sucrose, 0.01 M KCl, 0.001 M disodium salt of ethylenediamine tetraacetic acid, and 0.02 M phosphate at pH 7.5. Time moves from left to right and a downward deflection in the over-all curve indicates O_2 consumption. The numbers on the curves are the calculated rates of O_2 utilization in μmoles per L per sec. Temperature, 26 C. ADP = adenosine diphosphate.

and oxidative phosphorylation in the presence of α-ketoglutarate as substrate is shown in figure 1. The results of a control experiment without toxin are represented by a line superimposed over the experimental curve. In both cases the reaction mixtures were preincubated for 3 min with and without toxin. α-Ketoglutarate was then added and the rate of O_2 consumption recorded. In the presence of toxin, the rate of α-ketoglutarate oxidation was inhibited approximately 50 per cent as calculated from the slope of the curve. The addition of a phosphate acceptor, adenosine diphosphate (ADP), caused a 7- and 12-fold stimulation of respiration in the presence and absence of toxin, respectively, thus indicating the initiation of oxidative phosphorylation. The same rate of respiration during phosphorylation was observed in both cases. Approximately 20 seconds after the addition of ADP, respiration decreased indicating the conversion of the added ADP to adenosine triphosphate (ATP). From the amount of ADP added and the amount of O_2 consumed during the rapid phase of respiration (48 μmoles or 96 μatoms per L), a phosphorus to oxygen ratio of 2.9 was calculated in both experiments. A phosphorus to oxygen ratio of 2.8 to 3.2 was routinely observed in these sarcosome preparations with α-ketoglutarate as substrate. In summary then, the same rate and efficiency of oxidative phosphorylation was observed in the presence or absence of plague toxin, the toxin only affecting the rate of α-ketoglutarate oxidation.

The inhibition of α-ketoglutarate respiration in rat heart sarcosomes was studied as a function of time. These experiments (figure 2) showed increasing inhibition with time of preincubation with toxin. Under these conditions, succinate respiration was also inhibited but the per cent inhibition with the latter as substrate was considerably less than during α-ketoglutarate oxidation. In other experiments with rat heart preparations, inhibition of glutamate, β-hydroxybutyrate, and malate respiration were also demonstrated. By increasing the time of preincubation with toxin it was possible to show inhibition with decreasing amounts of toxin. Thus in figure 2, a preincubation time of 45 min with 1.0 mg of toxin was required for 100 per

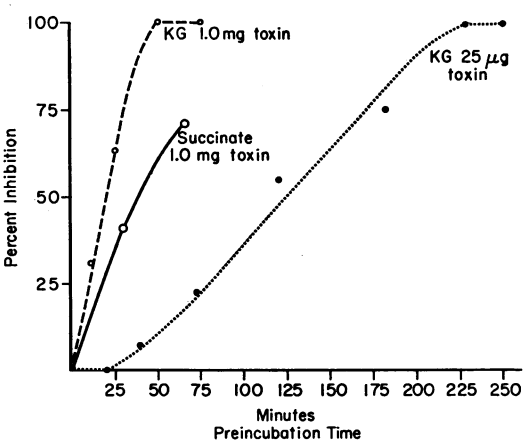

Figure 2. Time course of plague toxin inhibition of α-ketoglutarate (KG) and succinate respiration in rat heart sarcosomes. The reaction mixture contained 1.81 mg sarcosomal protein per ml and toxin as indicated. The other conditions are the same as described in figure 1, except that 10 ml of the reaction mixture was shaken in flasks in a Dubnoff shaker at 26 C. At varying time intervals, a 1-ml sample of the reaction mixture was removed from the flasks and placed in the cuvette of the O_2 electrode, and rates of respiration on the substrates tested. The per cent inhibition was calculated by comparison with controls incubated in the absence of toxin.

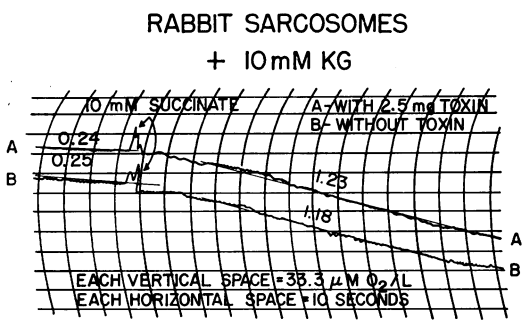

Figure 3. Effect of plague toxin on rabbit heart sarcosome respiration. The reaction mixture (1.0 ml) contained approximately 1.50 mg sarcosomal protein; other conditions as described in figure 2. Plague toxin (2.5 mg) was preincubated with one reaction mixture for 3 min before testing respiration; the control was incubated without toxin.

cent inhibition of α-ketoglutarate oxidation, whereas with 25 μg of toxin, 225 min were required to obtain the same inhibition.

In order to compare the action of plague toxin on mitochondrial preparations from different sources, it was necessary to standardize the toxin challenge. Selecting a 3-min preincubation time, inhibition of respiration of rat mitochondria could be demonstrated immediately upon addition of substrate when the concentration of the toxin varied between 0.5 and 5.0 mg per ml with the dilution of the mitochondrial suspensions used in these experiments. In succeeding experiments, a 2.5 mg amount of toxin per ml, preincubated for 3 min, was used because these conditions consistently showed 25 to 40 per cent inhibition of α-ketoglutarate respiration in the rat heart preparations.

Although rat heart sarcosome respiration was readily inhibited under these conditions, it was found that rabbit heart sarcosomes were surprisingly resistant to toxin action. In figure 3, two experiments are shown using rabbit heart preparations. It is clear that virtually identical rates of respiration obtain in the presence and absence of toxin. No inhibition in rabbit preparations was obtained if the toxin concentration was raised up to 5.0 mg per ml. This finding is of great interest since rabbits are resistant to the action of the toxin.

The above findings suggested an extension of the studies on species specificity of toxin action. Rat and rabbit heart preparations were compared with mouse heart sarcosomes; a summary of the data is presented in table 1. In all three sarcosome preparations examined, no effect of toxin was found on endogenous substrate respiration. Rat and mouse preparations showed comparable inhibitions of α-ketoglutarate and succinate respiration, whereas the oxidation of both of these substrates was unimpaired with the rabbit preparations. The per cent inhibition of substrate oxidation by toxin given in the table was not corrected for the slow endogenous respiration. If corrected in this manner, the inhibitions calculated are considerably higher than those given. For example, in rat sarcosomes, when endogenous respiration is subtracted from the respiration in the presence of α-ketoglutarate, a 50 per cent (rather than 37 per cent) inhibition is calculated when controls are compared with toxin treated experiments.

It should be mentioned that coupled phosphorylation during α-ketoglutarate respiration was unaffected after treatment with toxin in all three sarcosome preparations (figure 1). Rat and rabbit sarcosomes showed virtually the same phosphorylation efficiency (table 2, ADP/O values) and respiratory stimulation upon addition of ADP and 2,4-dinitrophenol, except in the presence of plague toxin where in the rat preparation the net rate of respiration was inhibited.

To test the specificity exhibited by plague toxin on mitochondrial respiration, the effect of two other bacterial endotoxins and bovine serum albumin (as control) on mitochondrial respiration was examined (table 2). The O antigen of *S. typhosa*, which is lethal to 3-kg rabbits in 20 to 50 μg amounts, did not affect respiration in either rabbit or rat heart sarcosomes. Likewise, the Vi antigen of *E. coli* did not effect mitochondrial respiration. Protein concentrations of bovine serum albumin as great as plague toxin were also not inhibitory.

Table 2 also shows a typical experiment dealing with the respiration of rat brain mitochondria where it was found that the toxin was not inhibitory. It can be seen from table 2 that only unaltered toxin is effective in inhibiting mitochondrial respiration. There is complete correlation between the toxicity of a toxin preparation for mice and its ability to inhibit the several oxidation reactions by the heart mitochondria. Specifically, when the toxin is denatured with heat so as to render it atoxic for the

TABLE 1

Species specificity of the effect of plague toxin on heart mitochondria

Source of Mitochondria	Toxin	O_2 Consumed			Inhibition		LD_{50} for Intact Animals
		Endogenous	α-Ketoglutarate	Succinate	α-Ketoglutarate	Succinate	
		μmoles/sec			%		*μg/kg body wt*
Rat	Absent	0.25 (13)*	0.84 (13)	2.35 (9)	—	—	60
	Present	0.24 (9)	0.53 (8)	1.84 (5)	37	22	
Rabbit	Absent	0.22 (15)	0.43 (19)	1.33 (14)	—	—	>5000
	Present	0.25 (7)	0.41 (11)	1.37 (5)	5	0	
Mouse	Absent	0.28 (14)	0.60 (14)	1.49 (7)	—	—	56
	Present	0.28 (6)	0.44 (6)	0.97 (6)	26	35	

Experimental conditions as described in figure 1 and text.

* The numbers in parentheses refer to the number of experiments for which the data were averaged. Statistical analysis of the data shows that probability varies between 0.01 to 0.001 by the T-test and is 0.001 by analysis of variants.

TABLE 2

Effect of plague toxin, Vi, and O antigen and bovine serum albumin on mitochondrial respiration and phosphorylation

Source of Mitochondria	Additions	O_2 Consumption				Adenosine diphosphate/O
		α-Ketoglutarate	Adenosine diphosphate	2,4-Dinitrophenol	Succinate	
		μmoles/L/sec				
Rat heart (2.21 mg protein)	None	0.72	1.93	2.65	—	3.2
	Boiled plague toxin (2.5 mg)	0.66	—	—	—	—
	Bovine serum (2.5 mg)	0.72	—	—	—	—
	O antigen (2.5 mg)	0.[illegible]	1.95	2.30	—	3.2
	Plague toxin (1.0 mg)	0.24	0.88	—	—	3.2
Rabbit heart (1.39 mg protein)	None	0.41	1.77	2.40	—	3.1
	Vi antigen (2.5 mg)	0.41	1.64	—	—	3.1
	O antigen (2.5 mg)	0.50	1.90	—	—	3.1
	Plague toxin (2.5 mg)	0.44	1.67	—	—	3.1
Rat brain* (6.10 mg protein)	None	0.34	—	—	1.10	—
	Plague toxin	0.35	—	—	1.10	—
Rat brain (6.10 mg)	None	0.60	—	1.13	—	—
	Plague toxin (2.5 mg)	0.60	—	1.10	—	—

Experimental conditions as described in figure 1 and text.

* These experiments were carried out in the same manner as the heart sarcosomes except that the molarity of sucrose was 0.25 M. Where 2,4-dinitrophenol was used, the concentration was 100 μM.

mouse or rat, it also loses its ability to inhibit exogenous mitochondrial respiration.

DISCUSSION

The inhibitory effect of plague murine toxin on the respiration of mammalian mitochondria suggests that one of its effects in living cells may be at the mitochondrial level of organization. Moreover, the action of the toxin on mitochondria varies with regard to species of animals from which the mitochondria are prepared as well as with regard to the organ from which they are

derived. It is interesting to note that this variation closely parallels the susceptibility and resistance of the animals or organs to the action of plague toxin. Thus, the hypothesis is suggested that the susceptibility of the rat and mouse to the action of the toxin is related to the ability of the toxin to inhibit the respiration of rat and mouse heart mitochondria whereas the reverse is true in the toxin-resistant rabbit. The test for this hypothesis may come from studies of effects of plague toxin on the functional activity of organ systems of different species. From the findings to date with the action of plague toxin on mitochondria, one would expect from the above hypothesis that there would be malfunction of the heart as an early sign of plague toxin action in a susceptible species. On the other hand, dysfunction of the brain would not be expected as a toxic manifestation under the proposed hypothesis. However, lack of sign of brain dysfunction may be due to lack of accumulation of the toxin in the brain (Ajl, Rust, and Woebke, *unpublished data*) in contrast to its deposition in heart muscle. The elucidation of any relation that may exist between the observed accumulation of plague toxin in certain tissues, and the susceptibility of mitochondria from those tissues to the action of plague toxin, must await further studies.

Of particular interest is the finding that unlike the results with cell-free extracts of bacteria, only unaltered toxin is effective as a depressant of mitochondrial respiration. Thus, if the toxin is treated in such a manner as to make it atoxic *in vivo*, it will no longer inhibit mitochondrial respiration when measured *in vitro*. This finding suggests that at least one of the mechanisms by which the plague toxin may kill an animal is via an inhibition of certain of its respiratory mechanisms. Two other bacterial toxins, i. e., the typhoid O and coli Vi antigens, did not inhibit mitochondrial respiration of rat or rabbit heart preparation when tested in equivalent concentrations. Moreover, the action appears not to be due to a nonspecific protein since equivalent amounts of bovine serum albumin do not influence the rate of O_2 consumption.

It may be pertinent to point out that the concentration of plague murine toxin generally employed, i. e., 2.5 mg, corresponds to a concentration of 3.57×10^{-5} M assuming the molecular weight to be 70,000 (Ajl *et al.*, 1955). On this basis, the effective concentration of toxin examined ranged from 7.1×10^{-5} to 3.57×10^{-7} M in the experiments reported. These concentrations fall well within the range of a variety of biological inhibitors and it therefore may be of significance with respect to the mechanism of toxin action on mitochondrial respiration systems.

In conclusion, it should be pointed out that it is specifically the oxidation of these compounds that the toxin inhibits and not their closely associated phosphorylating mechanisms. Further, the endogenous respiration of the mitochondria is unaffected and the inhibition of respiration only becomes apparent on the addition of exogenous substrate. These findings lend further evidence to the specificity of the effect exhibited by plague murine toxin.

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SUMMARY

Purified plague murine toxin has been found to inhibit the respiration of heart mitochondria obtained from the toxin-sensitive rat and mouse, and not from the toxin-resistant rabbit. Only exogenous respiration is inhibited; endogenous respiration and oxidative phosphorylation are unaffected. This inhibition is specific, i. e., bovine serum albumin, the Vi and O lipopolysaccharide antigens, for example, were not found to interfere with mitochondrial respiration. A correlation exists between the toxicity of the toxin molecule and its ability to inhibit mitochondrial respiration. Thus, heat-treated toxin is atoxic, and also has lost its ability to inhibit mitochondrial respiration. A hypothesis is presented which, if established, may offer an explanation for the action of plague murine toxin *in vivo*.

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