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Site of Action of Plague Murine Toxin
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This work was completed at the Albert Einstein Medical Center in Philadelphia, Pennsylvania.
In investigations on the mechanism of action of plague murine toxin, it has been revealed that the toxin is capable of inhibiting the respiration of mitochondria isolated from toxin susceptible animals by complexing with a non-enzymatic protein, reported to be located in the membranes of mitochondria and to satisfy various criteria for a structural protein, and thereby altering the structural configuration of the multi-enzymatic unit of a mitochondrion. Studies on the effect of plague murine toxin on protein biosynthesis have shown that Toxin B, one of the two protein components of the plague toxin, inhibits the incorporation of C\textsubscript{14} leucine or C\textsubscript{14} phenylalanine in the presence of poly U into trichloracetic acid precipitable material by cell-free rat liver systems. This inhibitory effect can be observed with as little as 10\mu g of toxin. The longer Toxin B is preincubated with the supernatant fraction, the greater is the percentage inhibition. This is not the case when Toxin B is preincubated with rat liver ribosomes. The inhibitory effect is not due to a protease or a nuclease activity of the toxin. In order to be able to trace the fate of plague murine toxin after injection to toxin susceptible mice and rats, an attempt was made to label the toxin with a gamma emitting isotope such as Se\textsuperscript{75}-methionine. Examination of disc gel electrophoretic profiles of toxins A and B following the labeling procedures showed that in both cases one single protein could be seen and that practically all of the radioactivity was associated with these proteins. Both preparations retained high biological activity.
The investigations which have been supported by this contract have been concerned with the mechanism of action of 2 protein toxins produced by the bacterium Pasteurella pestis, the causative agent of the disease known as plague. Our earlier studies have indicated that the toxin alters a number of mitochondrial energized processes primarily in heart mitochondria isolated from toxin-susceptible animals, but not in heart mitochondria isolated from toxin-resistant animals. The effects observed include inhibition of mitochondrial respiration (1, 2, 3), promotion of large amplitude mitochondrial swelling (4) and inhibition of respiration-dependent ion accumulation by mitochondria (5).

I. Interaction of Plague Murine Toxin with Mitochondrial Structural Protein

We have shown that the inhibition of mitochondrial respiration by plague murine toxin is related to its ability to alter the structural configuration of the multi-enzymatic unit of a mitochondrion. It was demonstrated that the toxin is able to interact with a non-enzymatic protein reported to be located in the membranes of mitochondria and to satisfy various criteria for a structural protein (6). This interaction with structural proteins derived from bovine or rat heart mitochondria resulted in the loss of toxicity of the toxin as well as the specific diminution of the gel diffusion precipitation band exhibited by Toxin B, one of the two protein species comprising plague murine toxin. Ultracentrifugal analysis of solutions containing equimolar ratios of Toxin B and bovine heart mitochondrial structural protein dissolved in 0.1% sodium dodecyl sulfate, 0.05 M NaCl and 0.01 M phosphate buffer (pH 10.5) revealed that the original 6.6 sedimentation coefficient (S) peak of the toxin and the 2.3 S peak of the structural protein vanished and a new 4.8 S peak was observed (7). The same results were obtained when the molar ratio of structural to toxin was 2:1. These findings suggest that structural protein is capable of complexing with Toxin B.

II. Effect of Plague Murine Toxin on the Incorporation of Amino Acids into Polypeptides by Cell-Free Rat Liver Systems

Reports on studies of the mechanism of action of bacterial protein toxins other than plague murine toxin have in recent years increasingly implicated some of these toxins in alterations involving protein biosynthesis by cells of certain tissues. During the past few years we have initiated experiments to determine if plague murine toxin has any effect on protein biosynthesis.

Initial amino acid incorporation studies were conducted with microsomes and dialyzed 105,000 xg supernatant fluid utilizing a standard incorporation mixture similar to that described by Johnson et al. (8). With this system incorporation of C14 amino
acids into hot trichloracetic acid (TCA)-precipitable materials is linear for approximately 20 minutes at 37°C, and is essentially complete after 40 minutes. The incorporation of C₁⁴ amino acids was found to be highly sensitive to RNAase, puromycin and cycloheximide but virtually unaffected by large concentrations of purified DNase.

Investigations on the effect of varying concentrations of Toxin B on the incorporation of C₁⁴ leucine showed that when 10, 25, and 50 µg of toxin were incubated with 105,000 xg supernatant fluid for 20 minutes at 37°C prior to the initiation of the reaction, the rate of C₁⁴ leucine incorporation was inhibited 30,60 and 75%, respectively. Toxoid, formalin inactivated toxin, exhibits some inhibitory effect but approximately 10 to 20 times as much toxoid is needed to give an effect comparable to that of the active toxin. When C₁⁴ phenylalanine incorporation by our cell-free rat liver system was measured in the presence of the artificial messenger, polyuridylic acid (poly U), the total incorporation in the absence of toxin at each time interval was appreciably higher than was observed in the C₁⁴ leucine experiments. However, the percentage inhibition of incorporation in the presence of each concentration of toxin employed was essentially the same in both cases.

Experiments designed to determine whether or not the toxin has any effect on the reagents included in the reaction mixture such as ATP or the ATP-generating system rather than on protein biosynthesis per se were conducted by incubating all of the components of the reaction mixture exclusive of microsomes, supernatant fluid and C₁⁴ amino acid with or without toxin for varying periods of time at 37°C and then allowing incorporation to take place by adding back the deleted constituents of the system. It was found that C₁⁴ leucine was incorporated just as well in the presence of toxin as in its absence, indicating that the inhibitory effect of the toxin on amino acid incorporation into polypeptides is probably not due to any interaction of the toxin with any of the components of the incorporation mixture other than supernatant fluid or microsomes. Attempts were made to determine whether the time of preincubation of toxin with supernatant fluid is related to the extent of inhibition by the toxin of amino acid incorporation into polypeptides. In the absence of toxin, the incorporation of C₁⁴ leucine does not vary with the time of preincubation of supernatant fluid at 37°C. In the presence of toxin, however, the inhibitory effect of a given concentration of toxin on C₁⁴ leucine incorporation becomes more pronounced as the time of preincubation of the toxin with supernatant fluid is extended.

Experiments involving preincubation of the toxin with microsomes showed that although some inhibitory effect of the toxin could be noted, this did not vary with time of preincubation. The possibility exists that some supernatant fluid could have been associated with the microsomal pellets.

(2)
Since our cell-free rat liver protein synthesizing system is extremely sensitive to inhibition by very low concentrations of RNAase and RNAase is a frequent contaminant of many bacterial enzyme preparations, our Toxin B preparations were tested for RNAase activity by the procedure of Spahr and Schlessinger (9). After incubation of Toxin B alone, undialyzed 105,000 xg supernatant fluid alone or Toxin B together with supernatant fluid with C14 poly U and precipitation of the poly U with 60% cold ethanol, any C14 nucleotides or small oligonucleotides liberated by RNAase action were assayed as soluble radioactivity. The counts per minute (cpm) released following incubation of 145 pg of Toxin B with C14 poly U were almost identical to that of the reagent blank. While undialyzed 105,000 xg supernatant fluid exhibits considerable RNAase activity, the addition of 145 pg of Toxin B to this system did not increase the ethanol-soluble nucleotides liberated from C14 poly U. These results show that our Toxin B preparations are free from significant RNAase activity in addition to any factor that would liberate a latent RNAase in the supernatant fluid.

In order to be able to perform meaningful experiments involving the location of the site of inhibition by plague murine toxin of protein biosynthesis, cell-free systems from rat liver were assembled from better defined components in the particulate and soluble fractions. Amino acid incorporation studies were conducted with ribosomes purified essentially by the procedure of Weinstein (10) except that in the final stage the ribosomes were sedimented through a cushion of 10% sucrose in Tris buffer to which ammonium chloride, magnesium acetate and β-mercaptoethanol were added according to the procedure of Jones et al. (11). pH5 fractions were prepared by a modification of the procedure of Keller and Zamecnik (12) as described by Weinstein (10). Initial incorporation studies in the absence of toxin utilizing the incorporation mixture described by Weinstein (10) and the filter paper disc method of Mans and Novelli (13) for assaying radioactivity revealed that the above described purification procedures resulted in the removal of messenger RNA and transfer RNA (tRNA). Upon the addition of commercially available poly U and tRNA to a system containing the optimal ratio of ribosomes to pH5 fraction the incorporation of C14 phenylalanine into TCA precipitable material was appreciable and linear for approximately 60 minutes.

In the presence of 10, 25, and 50 pg of Toxin B the percentage inhibition of C14 phenylalanine incorporation amounted to 20, 60 and 85%, respectively. Toxoid, on the other hand, had essentially no effect since as much as 200 pg of the inactivated toxin inhibited amino acid incorporation by only 5 to 10%. These experiments involved the direct addition of the toxin or toxoid to the reaction mixture rather than preincubation with the pH5 fraction or the ribosomes.

(3)
III. Distribution of Plague Murine Toxins In Vivo.

While there have been numerous studies conducted concerning the physiology and mechanism of action of plague toxins, the target organ or organs involved during infection with the plague bacillus or following toxin injection is still unknown. It is of interest, therefore, to prepare pure radioactively labeled toxin and trace its fate after injection to toxin susceptible mice and rats. It was envisaged that the detection procedure could be simplified by labeling the toxin with a gamma emitting isotope such as Se$^{75}$-methionine. We have carried out initial experiments (14) involving the labeling of Toxin A and Toxin B with Se$^{75}$-methionine which was included in the growth medium (15). The toxic proteins A and B were isolated by electrophoresis on polyacrylamide gel slabs and analyzed by disc gel electrophoresis (16). After the gels were stained, they were scanned with a Gilford spectrophotometer equipped with a linear transport attachment. Examination of the electrophoretic profiles of toxins A and B revealed that in both cases one single protein could be seen and that practically all of the radioactivity was associated with these proteins. The specific radioactivity was calculated to be 300 cpm/g for Toxin A and 227 cpm/g for Toxin B. Both preparations retained high biological activity.
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