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Physical Changes in the Epsilon Prototoxin Molecule of *Clostridium perfringens* During Enzymatic Activation

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Enzymatic activation of *Clostridium perfringens* epsilon prototoxin removed a small basic part of the molecule, causing a slight change in molecular weight (32,700 to 31,200) and a large change in isoelectric point (from pH 8.02 into fractions of 5.36 and 5.74).

The epsilon toxin of *Clostridium perfringens* is produced in the form of an inactive prototoxin which is activated by proteolytic enzymes (for review see reference 2). It has been shown that a combination of trypsin and chymotrypsin causes maximal activation (4). Habeeb (1) found that activation of prototoxin with trypsin caused a profound change in electrophoretic mobility, which suggested that a basic peptide could have been removed from the prototoxin. This study aimed to investigate more fully the changes that take place on enzymatic activation.

Highly purified epsilon prototoxin, produced as described previously (5), was activated by incubating 100 mg of prototoxin, 4 mg of trypsin immobilized on *O*-(carboxymethyl)(CM)-cellulose (Merck), and 4 mg of chymotrypsin also immobilized on CM-cellulose (Merck) in 4 ml of 0.1 M phosphate buffer, pH 8.0, at 37°C for 30 min. The solution was filtered through a membrane filter (Millipore) and a column of Sephadex G-25, and the first peak eluted was lyophilized. Toxicity tests were performed as described previously (5) by injecting mice intravenously with doubling dilutions of toxin. The highest dilution of toxin that killed two of three mice within 24 h was considered a lethal dose (LD). Enzymatic activation increased specific toxicity of the prototoxin 1,000-fold to 19.8 × 10^6 LD/mg of N. Prototoxin solutions containing 0.16 mg of N/ml had an optical density (OD) of 1.01 (standard deviation, 0.05) at 278 nm. The absorbance-to-nitrogen content ratio did not change on activation. Because fractions from isoelectric focusing contained ampholytes, nitrogen determinations could not be done. The protein (N × 6.25) content was, therefore, estimated from the extinction coefficient $E^{1\%}_{278\,nm} = 10$. No correction was made for absorbance of the ampholytes, which is low at 278 nm.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Weber et al. (3) was used to estimate the molecular mass of prototoxin and activated toxin. Isoelectric focusing was done on a LKB 8101 Ampholine column containing a 40 to 0% sucrose gradient. The gradient contained 3 ml of either pH 7 to 9, 5 to 8, or 4 to 6 ampholytes (LKB) and 0.35 ml of pH 3 to 10 ampholytes. In one case pH 5 to 6 ampholytes, which were prepared by isoelectric focusing of pH 4 to 6 ampholytes, were used. Samples of prototoxin or activated toxin, containing 8 to 12 mg of protein, were applied to the middle of the gradient during gradient formation. The initial voltage applied across the gradient was 400 V, which was increased to 600 V after about 4 h, and this tension was maintained for a further 16 h. The voltage was then increased to 900 V for a further 2 h. The power was never allowed to rise above 2 W, and the column was cooled by pumping water at 4°C through the cooling jacket. Fractions of 2.5 ml were collected at the end of the run, and the pH of each fraction was measured soon after it was collected. The OD of each fraction at 280 nm was measured.

The major peak of epsilon prototoxin had an isoelectric point of 8.02 (range, 8.00 to 8.05 in three experiments; Fig. 1). The activated toxin had two major peaks. The amount of protein in the two peaks varied somewhat in different batches of toxin, but the peaks consistently focused at pH 5.36 (range, 5.25 to 5.50 in eight experiments) and at 5.74 (range, 5.65 to 5.80 in eight experiments). The material from both peaks was toxic for mice. The toxicity of the more acidic fraction was 6.4 × 10^6 to 12.8 × 10^6 LD/mg, whereas that of the more basic material was only 8.0 × 10^6 LD/mg (Fig. 2). The significance of minor peaks that are not toxic is unknown. Separation of the two peaks was im-

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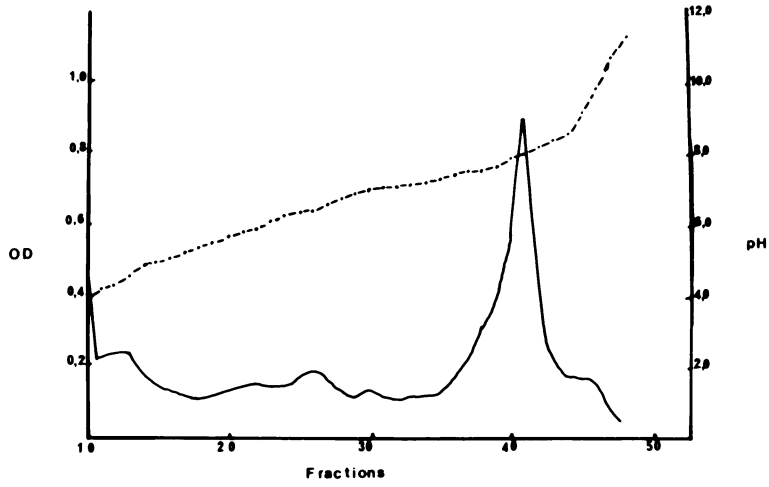

FIG. 1. *Isoelectric focusing of C. perfringens prototoxin on a pH 5 to 8 gradient. OD at 280 nm, ——; pH, — · — · .*

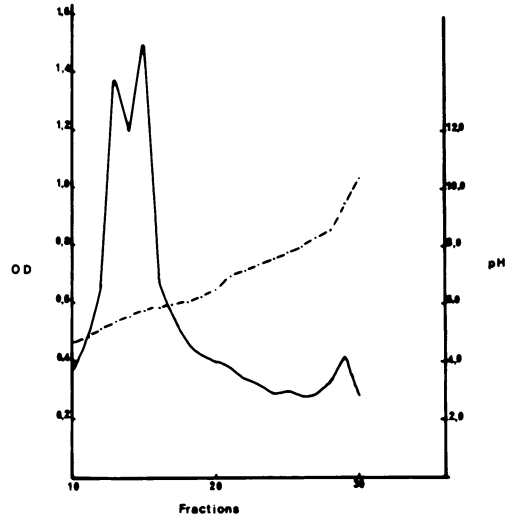

FIG. 2. *Isoelectric focusing of activated C. perfringens epsilon toxin on a pH 5 to 8 gradient OD at 280 nm, ——; pH, — · — · .*

proved when a shallower gradient was used (Fig. 3).

The main prototoxin band was estimated to have a molecular mass of 32,700 (range, 30,000 to 35,200 in three experiments). The toxin only showed one major band on SDS-gel electrophoresis, and the molecular mass was 31,200 (range, 29,000 to 34,000 in three experiments; Fig. 4). The differences in molecular mass were small, but in each experiment in which prototoxin, toxin, and markers were run in the same batch of polyacrylamide in the same electrophresis tank, the toxin was slightly smaller than the prototoxin. This method is not sufficiently precise to measure the small mass difference accurately. It does, however, indicate that a small fragment or a number of small fragments are removed from the prototoxin during enzymatic activation. The large change in isoelectric point during activation shows that the peptides removed must be highly basic in nature. Two toxic fractions were found after enzymatic activation.

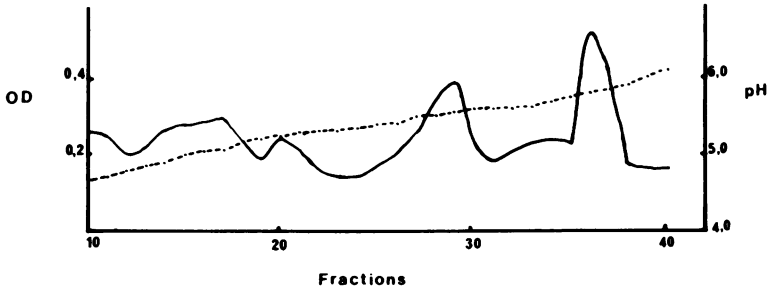

FIG. 3. *Isoelectric focusing of activated C. perfringens epsilon toxin on a pH 5 to 6 gradient OD at 280 nm, ——; pH, — · — · .*

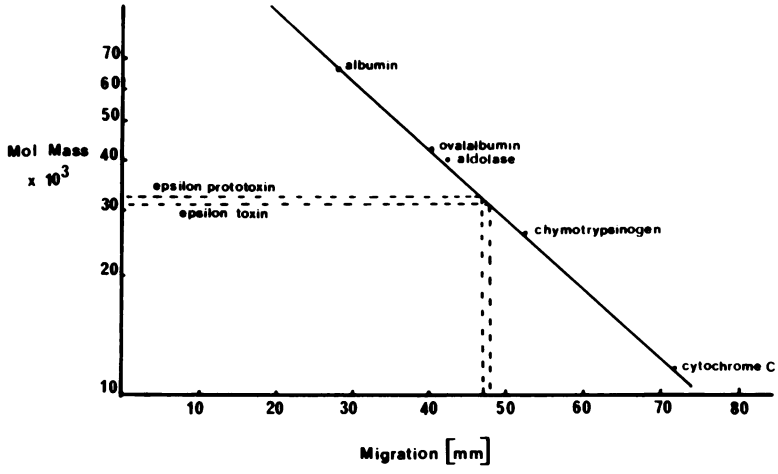

FIG. 4. *Plot of electrophoretic mobility against molecular mass of prototoxin, toxin, and marker proteins in SDS-polyacrylamide gel electrophoresis.*

The more acidic protein (pI, 5.36) was fully toxic, whereas the more basic protein (pI, 5.74) appeared to be only partially activated.

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