

## STUDIES ON PLAGUE

### I. PURIFICATION AND PROPERTIES OF THE TOXIN OF *Pasteurella pestis*

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The plague bacillus is known to contain several distinct and readily separable antigenic components. Two of these, the envelope antigen and the endotoxin, have been intensively investigated with particular regard to their role in immunity and infection (Pollitzer, 1954).

It is generally assumed that no soluble extracellular toxin is produced by *Pasteurella pestis*, but certain aspects of the symptomatology and pathology of the disease have long suggested that during infection there are toxic substances of considerable potency liberated from the cells (Dieudonné and Otto, 1928). The recent observations of McCrumb *et al.* (1953) that antibiotics failed to save patients treated late in pneumonic plague in spite of the control of the bacterial infection have renewed interest in the toxins of *P. pestis* and their role in this disease.

Studies dealing with the biological action and antigenic behavior of plague toxin have been handicapped by the difficulty of obtaining preparations of adequate purity. Nevertheless, it was known that the *P. pestis* toxin is contained within the cell and is readily obtained by lysis, sonic vibration, or by treatment with several simple compounds such as sodium chloride (Baker *et al.*, 1952), dilute alkali (Lustig and Galeotti, 1901), and sodium desoxycholate (Goodner *et al.*, 1955). The crude preparations of toxin, consisting largely of protein, are antigenic and immunogenic in certain rodents (Rowland, 1910), and are precipitated and partially purified by ammonium sulfate (Baker *et al.*, 1952) and calcium chloride (Zheltenkov, 1946). Such preparations, although relatively stable and highly toxic for mice, are very difficult to free of residual contaminating components (Baker *et al.*, 1952; Meyer, 1949; Meyer *et al.*, 1949). Recently Englesberg and Levy (1954) obtained fractions which were highly toxic by utilizing autolysates of *P. pestis* grown at 30 C in semisynthetic media. Although the relative purity of this material is not indicated, it appears

to provide a source of greater yield of toxin than the previous methods.

The present study has utilized several chemical fractionation procedures combined with continuous paper electrophoresis to obtain a toxic protein from *P. pestis* which appears to be relatively homogenous, highly antigenic, and free of envelope antigen. Its chemical, physical, and immunological properties will be described in detail in this and following reports.

#### MATERIALS AND METHODS

*Preparation of Pasteurella pestis.* Since both virulent and avirulent strains of the plague bacillus produce a potent toxin, to facilitate this work, the avirulent "Tjiwidej" (TJW) strain of *P. pestis* was used. Large amounts of bacterial cells were obtained by the following procedure. A 500 ml bottle of trypticase soy broth (BBL) was inoculated with the growth from a 24-hour-old trypticase soy agar slant and incubated overnight at room temperature. This seed culture was then added to 16 liters of broth, which was incubated with constant, gentle aeration for 24 hours at room temperature. The organisms were sedimented by Sharples centrifugation, washed twice with distilled water, and resuspended in a small volume of water. For rapid desiccation of the cells they were then aspirated into 4 volumes of acetone which was cooled to approximately  $-70^{\circ}\text{C}$  in a dry ice bath. Following this step the acetone-cell mixture was removed from the bath and allowed to stand 30 to 60 minutes. The cell mass was collected either by filtration or centrifugation and resuspended in 4 volumes of chilled acetone. To dehydrate the cells thoroughly this procedure was repeated 3 times, following which the residual acetone was removed *in vacuo*. Dried cells which were not used immediately were stored in the refrigerator at 4 to 6 C. It may be noted that such storage of the dry cells for many months did not appreciably affect the quantity of extractable toxin.

*Determination of toxicity of P. pestis toxin.*

Toxicity was measured by intraperitoneal or intravenous injection of 14 to 18 g albino Swiss mice with 0.5 ml amounts of twofold serial dilutions of the toxin preparation in physiological saline. Eight mice were used for each dilution and were observed twice daily for 48 hours. The LD<sub>50</sub> was calculated on the basis of deaths occurring during this interval. Following intraperitoneal inoculation deaths occurred as early as 3 hours after the injection of multiple LD<sub>50</sub>'s of toxin, and even with smaller doses only an occasional death was observed after 24 hours. Although somewhat less sensitive than the intravenous, because of its relative simplicity, the intraperitoneal route was used for routine titrations of toxin preparations.

*Serologic procedures.* Antisera against purified toxin were prepared by the subcutaneous injection of two groups of rabbits with mixtures of equal parts of Freund's adjuvant (Freund *et al.*, 1948) and toxin. One group received 1.0 mg. and the other 10.0 mg of toxin, respectively. Four weeks later all animals were given a booster dose of 0.5 mg of toxin intravenously. Two weeks following this last dose sera were obtained, tested, and found to possess marked toxin neutralizing, hemagglutinating, complement fixing, and precipitating activity. A detailed description of the serological procedures used in the current work is given in another article in this series (Warren *et al.*, 1955).

*Analytical methods.* Nitrogen was determined by the micro-Kjeldahl method. Phosphorus was estimated colorimetrically (Gomori, 1942); sulfur, gravimetrically after dry combustion of the material (Johns, 1941). Carbohydrates were determined colorimetrically (Morris, 1948) and material absorbing at 260 m $\mu$  (considered as nucleoprotein and nucleic acids) in the Beckman spectrophotometer. Electrophoretic mobilities were established at 0 to 1 C by moving boundary methods in a Tiselius apparatus (Perkin-Elmer).<sup>1</sup> Diffusion constants were determined in Tiselius cells at 20 C.<sup>1</sup> Overnight equilibration against 5 volumes of barbital buffer was employed prior to the electrophoretic or diffusion experiments. Ultracentrifugal analyses were performed in the Spinco ultracentrifuge, Model E, using the

<sup>1</sup>We are indebted to Miss Pearl Anderson, Department of Biochemistry, Army Medical Service Graduate School, for the performance of these determinations.

Philpot-Svensson schlieren optical system and 0.8 ml cells.

#### EXPERIMENTAL RESULTS

*Chemical purification of the toxin.* Baker and coworkers demonstrated that a *P. pestis* protein which was extracted from acetone-dried organisms with 2.5 per cent sodium chloride and the material obtained after fractionation with ammonium sulfate was toxic but contaminated with atoxic soluble antigens.

The rather lengthy method used at present utilizes the initial steps described by Baker *et al.* (1952) and is summarized in table 1. A typical experiment consisted of suspending 20 g of acetone-dried bacilli in 400 ml of 2.5 per cent sodium chloride solution saturated with toluene and allowing it to stand for 24 hours at room temperature. The cells were then removed by centrifugation and re-extracted in a similar manner using 200 ml of the sodium chloride solution. The combined extracts were dialyzed overnight against cold distilled water. After dialysis, solid ammonium sulfate was added in the cold with constant stirring to 0.20 saturation, and the mixture allowed to stand for several hours in the refrigerator. The precipitate which formed was removed by centrifugation, found to be essentially nontoxic, and discarded. Solid ammonium sulfate was again added to the supernate with constant stirring in the cold to 0.6 saturation. After standing several hours in the refrigerator, the precipitate was collected, dissolved in cold distilled water, and dialyzed until free of salt; this fraction was rich in toxin. Ammonium sulfate fractions obtained above 0.6 saturation were found to be essentially nontoxic.

To facilitate the separation of contaminating envelope substance from the material obtained between 0.2 and 0.6 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation, 0.05 N HCl was added to the salt-free solution dropwise with constant stirring in the cold to a pH of 4.7 to 4.8, at which acidity the bulk of the toxin was precipitated. The precipitate was centrifuged off, taken up in several volumes of distilled water, and brought into solution by adding 0.1 N NaOH to a pH of 7.0. This procedure was repeated twice. The solution was then dialyzed overnight against distilled water in the cold. A small aliquot was removed to determine the dry weight of the material at this stage. Following this, sufficient 1 N MnCl<sub>2</sub> was added to give a ratio of 1 ml of the MnCl<sub>2</sub> solution for each 150

TABLE 1  
*Procedures in the chemical fractionation of Pasteurella pestis toxin*

Procedure	Purpose	Yield Toxic Fraction	Intraperitoneal LD <sub>50</sub>	Total Units of Toxin*
1. Sodium chloride extraction (2.5%)	Extraction of toxin from acetone-dried bacilli	mg 4,835	μg 18.0	268,610
2. Ammonium sulfate precipitation (0.2-0.6 sat.)	Precipitation and concentration of toxin	2,113	8.2	257,682
3. Isoelectric precipitation at pH 4.7	Partial separation of toxin from envelope substance	1,307	8.3	157,470
4. Treatment with manganese chloride	Removal of nucleic acids	420	7.4	56,700
5. Methyl alcohol precipitation	Concentration of protein and removal of extraneous materials	408	7.0	57,300
6. Calcium phosphate gel absorption and elution	Further separation of toxin from envelope substance	267	6.5	35,100
7. Chloroform extraction	Removal of lipoidal materials	215	4.1	33,000
8. Final isoelectric and ammonium sulfate precipitation	Final concentration of toxin and further removal of extraneous materials	~115	2.6	~30,000

$$* \text{ Units of toxin} = \frac{\text{Total dry weights, } \mu\text{g}}{\text{LD}_{50}}$$

mg dry weight of the material in the solution. The precipitate which formed was centrifuged off and discarded, for it contained little or no toxin. Excess  $\text{MnCl}_2$  was removed from the supernate by dialysis. Whereas nucleoprotein-like materials were present in the toxic solution, as seen by its absorption spectrum prior to the addition of  $\text{MnCl}_2$ , a typical protein absorption curve is obtained after this treatment (figure 1).

Methanol was employed to precipitate the toxin in the following manner: To the supernate, after  $\text{MnCl}_2$  treatment, was added one-half volume of 90 per cent methanol in the cold. The formed precipitate was allowed to stand one hour in the refrigerator, centrifuged, and one-half volume of methanol again added to the supernate. The precipitates were collected in several volumes of distilled water and brought into solution on the addition of a few drops of

0.1 N NaOH. The supernate after the second alcoholic extraction was found to be nontoxic and was discarded.

To remove residual contaminating materials and particularly envelope substance, the dissolved and dialyzed alcohol precipitate was treated with 4 ml of calcium phosphate gel per 100 mg of dry weight of the toxic solution. The gel was allowed to remain in contact with the toxin for 2 hours in the cold after which the mixture was centrifuged and the supernate discarded. The toxin is adsorbed onto the gel whereas enveloped substance is not (Reedal, 1952, unpublished observation). The toxin was eluted from the calcium phosphate by adding a small amount of 0.2 M phosphate buffer containing 2.5 per cent sodium chloride and allowing the mixture to stand for two hours in the cold. The gel was removed by centrifugation, and the

supernatant solution dialyzed overnight in the cold.

Free lipids and lipoproteins were removed by shaking the solution with two volumes of chloroform intermittently in the cold for one hour in a separatory funnel. The chloroform layer was discarded, and the aqueous layer dialyzed and concentrated by subsequent isoelectric and ammonium sulfate precipitations. The product thus obtained still contained impurities as indicated by ultracentrifugal analysis. These were in part removed by centrifugation in an angle ultracentrifuge (Spinco) at  $105,400 \times G$  for 120 minutes. However, the supernates when observed in the analytical ultracentrifuge still contained one major component and frequently one or more minor components.

*Final purification of the toxin by continuous paper electrophoresis.* The high resolving power for fractionating protein mixtures afforded by continuous paper electrophoresis led to the employment of this technique for final purification of the toxin. The cell and method used are a modification of that described in detail elsewhere (Durrum, 1951a). However, since this procedure has not hitherto been applied to the purification of bacterial toxins, a brief description of it follows. A filter paper curtain is suspended from the edge of a plastic trough containing electrolyte. The sheet is cut in such a manner that its outer lower edges afford two tabs which dip into the electrode vessels. Between the electrode tabs, the bottom edge is serrated to provide drip-points. At some position near the top edge of the curtain a small tab is produced by making two parallel vertical cuts and one horizontal cut to free a small section which is bent forward. This serves as a wick by means of which the mixture to be separated is continuously fed by capillarity to the filter paper curtain. Electrolyte continuously "siphons" onto the paper from the plastic trough and passes down the curtain. An electric potential is applied across the filter paper curtain resulting in deviation of the various mobility species toward the anode or cathode.

Figure 2 illustrates the patterns obtained when crude, partially purified and purified *P. pestis* toxins are subjected to paper electrophoresis. The mixture being separated in figure 2A was a saturated solution containing approximately 35 to 50 mg of crude toxin. This was fed by a

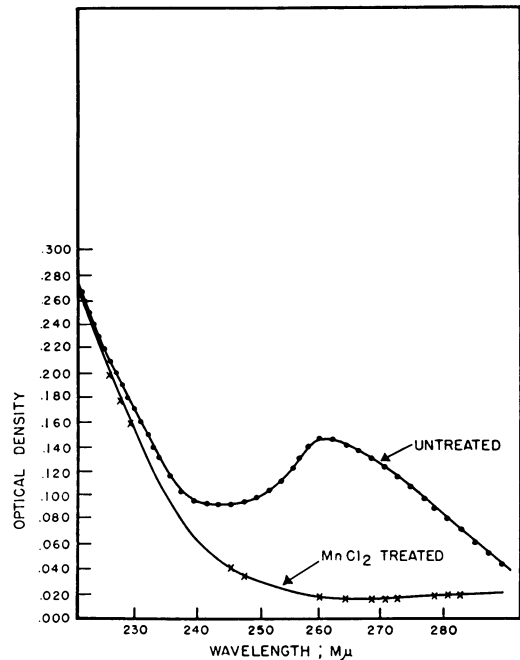


Figure 1. Absorption spectra of *Pasteurella pestis* toxic solutions before and after  $MnCl_2$  treatment.

2 mm wide filter paper wick to a tab located in the center of a sheet of Schleicher and Schuell (S and S) no. 589 filter paper measuring 30 cm across. The electrolyte was 0.025 M barbital buffer, pH 8.6. With the cell at room temperature a potential of 500 volts was applied. Within 60 minutes, when a steady state had been established, a current of about 12 milliamperes was observed which remained constant throughout the various experiments. The patterns shown in figure 2 were obtained after current had been applied for approximately 18 hours. The curtains were then dried in an oven and stained with bromphenol blue (Durrum, 1951a). Proteinaceous materials stain blue by this procedure and appear as black patterns in the photographs. Figure 2B shows the appearance of the product following the chemical fractionation procedure described in the preceding paragraphs. Inspection of this figure reveals one deeply colored zone at drip-point no. 9 and a rather wide lighter colored zone extending from drip-points no. 5 to no. 8. Titration of the various fractions revealed that only the one collected at drip-point no. 9 was toxic. However, the chemically fractionated toxin

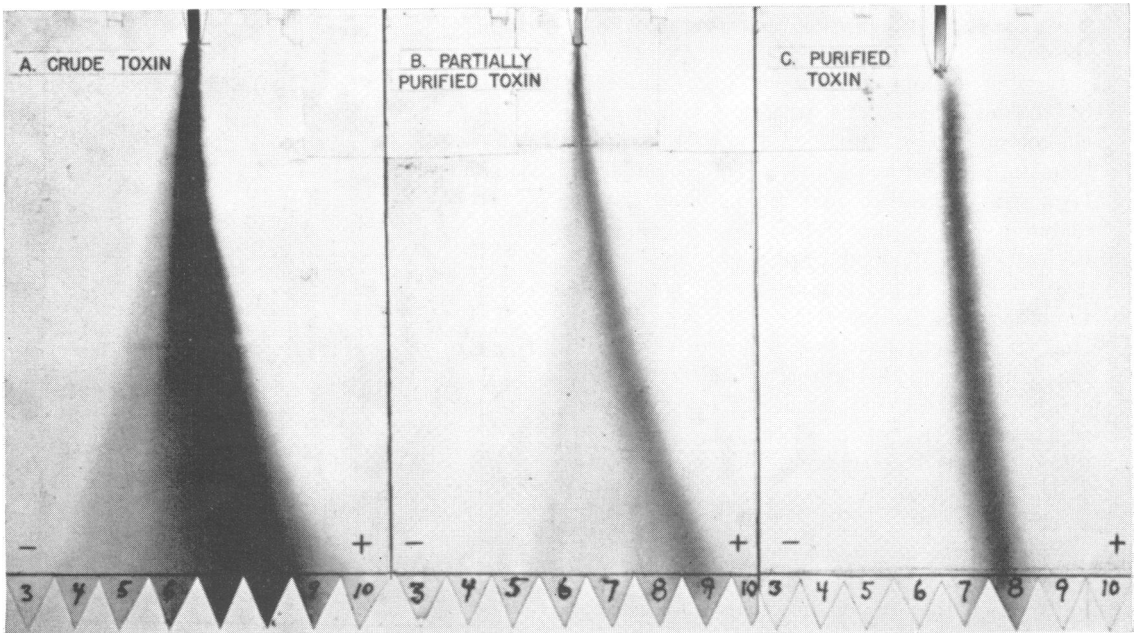


Figure 2. Paper electrophoretic patterns of crude, partially purified and purified toxic preparations of *Pasteurella pestis*. Paper: S and S no. 589; current: 10-15 MA; voltage: 500 v; buffer: barbital, 0.025 M.

still contains proteinaceous impurities as evidenced by its broad and diffuse pattern. That considerable purification has been achieved by chemical means can be seen when figure 2B is compared with figure 2A which represents a crude sodium chloride and ammonium sulfate precipitated toxic preparation. Purified toxin (figure 2C) was obtained by passing chemically fractionated toxin through the paper electrophoresis cell, dialyzing, and concentrating it by freeze-drying and then passing it through the cell for a second time. The final material was again dialyzed to remove buffer, freeze-dried, and stored in the dry state. Examination of figure 2C shows that there are no indications of any contaminating proteinaceous substances. Only the material collected at a single drip-point (no. 8 in this instance) was toxic. The toxin thus obtained is pale yellow in color in a 0.5 per cent solution in distilled water. The solubility of the pure toxin is approximately 5 to 8 mg per ml. Its refractive index is 1.33392. The purified toxin contains no characteristic absorption band either in the visible or ultraviolet regions of the spectrum.

*Immunological analysis of purified P. pestis toxin.* (Purified toxin hereafter refers to material purified by the chemical and electrophoretic

procedures described.) Indication of the antigenic homogeneity of the purified *P. pestis* toxin has been obtained by determining its reactions with different types of antisera using a variety of techniques. In addition, a potent specific antitoxin has been prepared, and this has been tested for its reactivity with a variety of *P. pestis* somatic and capsular antigens. These studies indicate that the purified toxin contained no demonstrable capsular antigens. It reacted with its antitoxin by complement fixation, flocculation, and hemagglutination procedures. Similarly, the antitoxin, although capable of neutralizing the toxins of several other *P. pestis* strains, did not react with capsular or envelope materials. A detailed description of these studies is given in another article in this series (Warren *et al.*, 1955).

*Biophysical analysis of purified P. pestis toxin.*  
*Paper electrophoresis.* Paper electrophoresis was carried out with a number of different buffers in order to determine whether the toxin migrated homogeneously at various pH's. Solutions for analysis were prepared by dissolving 6 mg of purified toxin, Lot no. 8, and allowing it to migrate on S and S no. 589 filter paper, as previously described, long enough to obtain a pattern. Figure 3 is a photograph of the migration pattern of the toxin at pH 4.0 in acetate, pH

6.0 and 8.0 in phosphate, and pH 10.0 in borate. From the consistent narrow migration bands obtained at each pH, it appears that the toxin is free of appreciable amounts of proteins of other mobilities.

*Electrophoresis.* The electrophoretic mobilities were determined for two lots (no. 6 and no. 7) of purified toxin. One-half per cent solutions were equilibrated overnight against several changes of barbital buffer, 0.1 ionic strength at pH 8.5. Following this, the toxin and buffer were placed in the Tiselius cell and migration observed for approximately 9,000 seconds. The electro-

phoretic pattern of Lot no. 7 is shown in figure 4. The toxin contained only one sharp boundary with mobilities of: ascending  $7.44 \times 10^{-5}$  cm<sup>2</sup>/volt-sec; descending  $6.99 \times 10^{-5}$  cm<sup>2</sup>/volt-sec.

*Diffusion.* In order to estimate the molecular weight the diffusion constant was determined using purified *P. pestis* toxin, Lot no. 7, in barbital buffer of pH 8.5 as above and at protein concentrations 0.98 per cent, 0.78 per cent, and 0.51 per cent as determined from areas of the diffusion curve. The temperature of the bath was 20 C, and diffusion was observed for the times shown in figure 5. Since the diffusion of

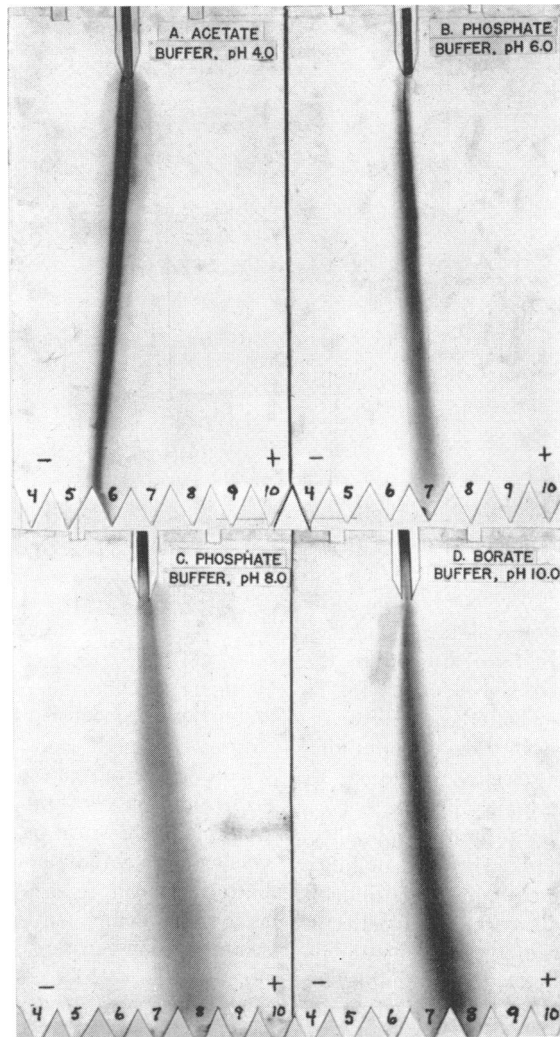


Figure 3. Paper electrophoretic patterns of purified plague toxin at various pH's. Paper: S and S no. 589; current: 10-15 MA; voltage: 500 v; buffer: acetate, 0.0140 M, pH 4.0; phosphate, 0.0096 M, pH 6.0; phosphate, 0.0400 M, pH 8.0; borate, 0.0500 M, pH 10.

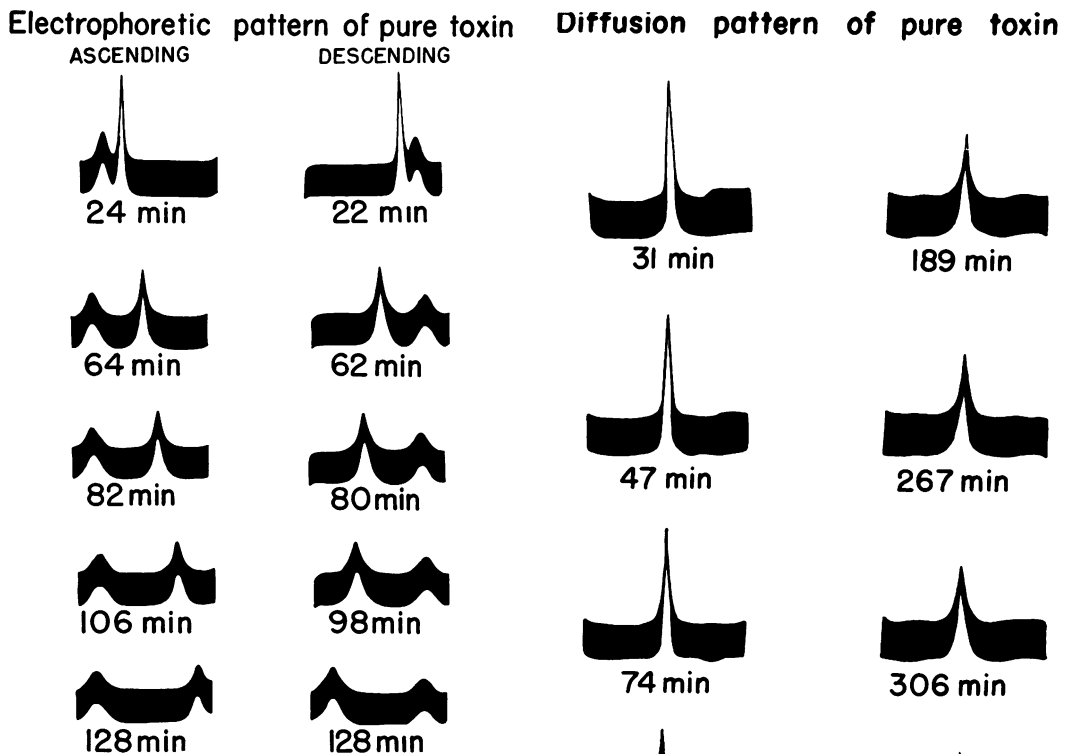


Figure 4. Moving boundary electrophoretic pattern of purified toxin Lot no. 7 derived from *Pasteurella pestis*.

this material appeared to be independent of the toxin concentration within the range indicated above, the results were averaged and a diffusion constant of  $3.43 \times 10^{-7}$  cm<sup>2</sup>/sec obtained in barbital buffer at 20 C.

**Ultracentrifugation.** Several lots of purified *P. pestis* toxin that have been studied in the analytical ultracentrifuge were found to be uniformly free of secondary boundaries. However, difficulties have been encountered in obtaining sedimentation data by either refraction or absorption methods. The toxin appears to be a relatively small molecule of limited solubility. Even at a slightly alkaline pH, when the solubility of the material is at a maximum, it is difficult to obtain concentrations above 0.8 to 1.0 per cent. Further, such solutions are of low viscosity and do not absorb well in wavelengths available for sedimentation analysis. All of the purified preparations examined gave low refractive boundaries, and these tended to flatten and spread during the run. In experiments, using two different batches of purified toxin, it has been possible to obtain

Figure 5. Diffusion pattern of purified plague toxin Lot no. 7 derived from *Pasteurella pestis*.

adequate serial photographs from which a sedimentation constant could be calculated. An example is shown in figure 6 which represents a tracing of an actual photograph to accentuate the pattern. It can be seen that the toxin migrated as a single component throughout the run. The sedimentation constant in distilled water for Lot no. 6 is  $S_{20}^w = 2.73 \times 10^{-13}$ .

**Chemical and physical properties of purified toxin.** The purified toxin dissolves slowly in water or neutral buffer solutions to give a slightly viscous, opalescent solution with a pale yellow color. The isoelectric point of the material is

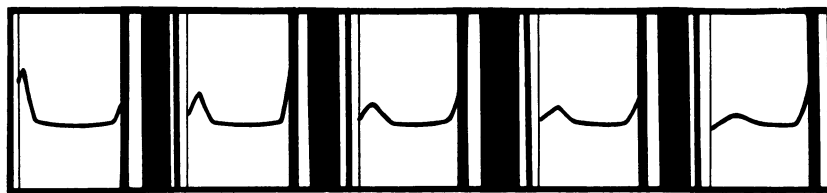


Figure 6. The ultracentrifuge pattern of purified plague toxin Lot no. 6. 0.6 per cent solution of toxin in distilled water. Centrifugal field,  $159, \times 700$  G.; bar angle, 45 degrees; temperature, 26.5 to 27.5 C; pictures at 16 minute intervals.

4.7. A summary of certain chemical analyses is presented in table 2. Judging from the chemical and physical characteristics as well as the nitrogen content (14 per cent) the toxin appears to be a protein which contains a significant amount of sulfur. The preparations are free of carbohydrate and phosphorus. Nucleoproteins are not detectable in the absorption spectrum of the material.

Extensive investigation has been made of the hydrolysis products of purified *P. pestis* toxin. Figure 7 is a photograph of a typical pattern produced by the toxin after hydrolysis for 24 hours in 6 N HCl and the filter paper dried and stained with ninhydrin. Although it does not indicate either the number or identity of the components, it does show the relative concentration of the basic, acidic, and neutral amino acids. Using standard ionophoretic procedures (Durrum, 1951b), however, at least 12 amino acids were obtained and identified. These included: arginine, alanine, glycine, serine, threonine, cystine, tyrosine, methionine, proline, aspartic and glutamic acids, and leucine. (Since isoleucine and valine have the same mobility as leucine under the experimental conditions that were employed, the final identity of the last amino acid remains to be established.) In addition to these amino acids at least three unknown components were obtained in chromatograms after hydrolysis. One of these components fluoresces; the other absorbs in the ultraviolet. The third is an uncommon ninhydrin-reacting substance. The actual location of the unknown components with respect to the known amino acids is illustrated on the chromatogram shown in figure 8.

#### Toxicity and stability of purified *P. pestis* toxin.

The LD<sub>50</sub> of *P. pestis* toxin in the mouse is dependent upon the route of inoculation as well as upon the use of certain compounds, *e.g.*, sodium desoxycholate, which are capable of

potentiating its toxicity (Goodner *et al.*, 1955). It should be noted that the intraperitoneal LD<sub>50</sub> in  $\mu\text{g}$  of the crude extracts is approximately 18 (see table 1). Following the chemical fractionations the LD<sub>50</sub> has decreased to approximately 3  $\mu\text{g}$ . The average LD<sub>50</sub> of 6 lots of electrophoretically purified toxin was 8  $\mu\text{g}$  by the intraperitoneal and 3  $\mu\text{g}$  by the intravenous route. Some toxicity is lost when the preparation is passed through the electrophoresis cell, and the LD<sub>50</sub> of the final purified toxin is invariably above that of the material obtained at the end of the chemical fractionation procedures. The cause for this partial inactivation of the material has not yet been determined.

The stability of purified toxin as compared with that of crude preparations has been established by determining its intraperitoneal mouse

TABLE 2

Chemical and physical properties of purified plague toxin

Nitrogen content.....	14.4%
Phosphorus content....	<0.2%
Sulfur content.....	1.9%
Carbohydrate content..	0
Molisch reaction.....	Negative
Anthrone reaction....	Negative
Carbon.....	46.47%
Hydrogen.....	6.62%
Isoelectric point.....	4.7
Electrophoretic mobility	
Ascending.....	$7.44 \times 10^{-5}$ cm <sup>2</sup> /volt-sec
Descending.....	$6.99 \times 10^{-5}$ cm <sup>2</sup> /volt-sec
Sedimentation constant	$S_{20}^w 2.73 \times 10^{-13}$
Diffusion constant.....	$3.43 \times 10^{-7}$ cm <sup>2</sup> /sec
Refractive index.....	1.33392
Approximate molecular weight.....	70,000



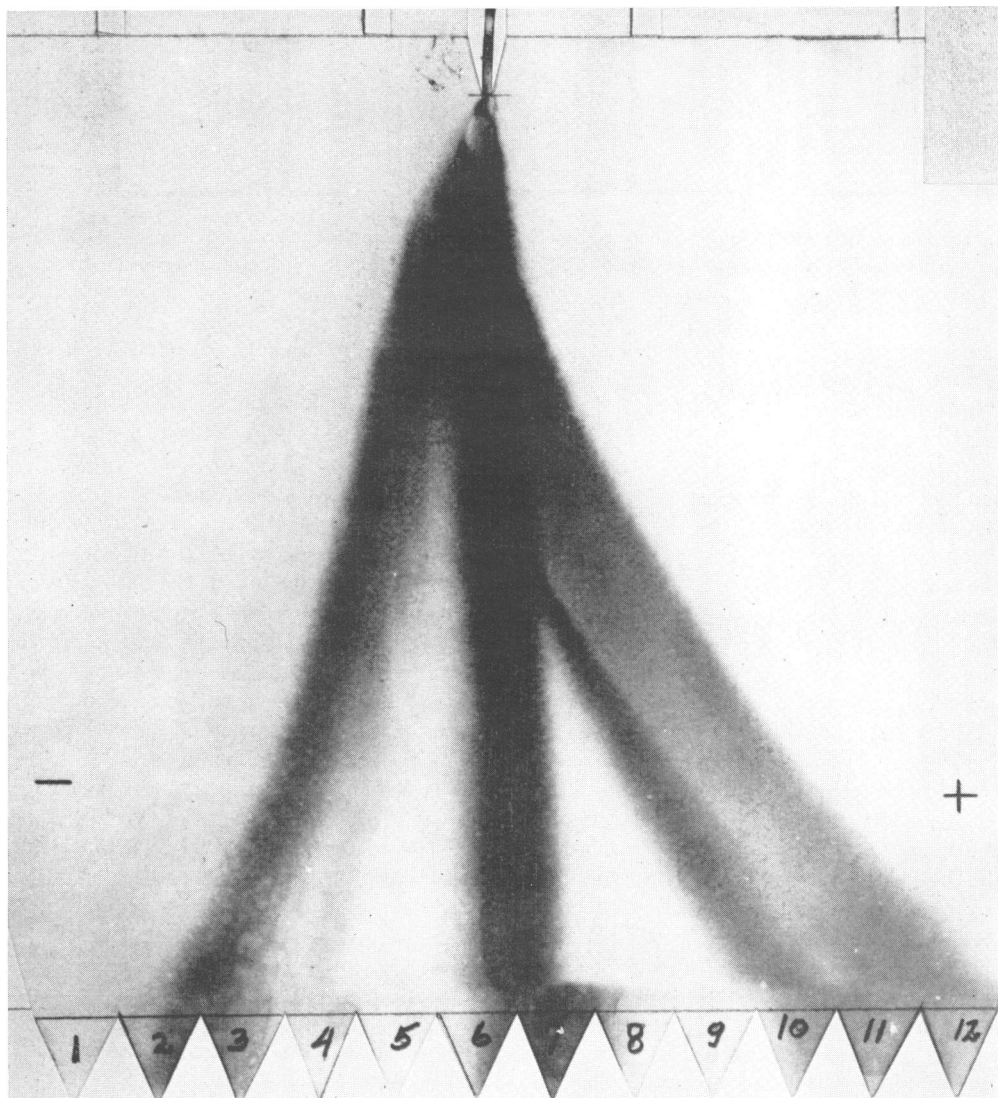


Figure 7. Paper electrophoretic pattern of acid hydrolyzed *Pasteurella pestis* toxin

toxicity after intervals of storage under various conditions. The toxicity of both purified and crude preparations remains quite stable when toxin solutions are stored at low temperatures. Table 3 provides data on the stability of toxin at various temperatures and in two solvents. At  $-20^{\circ}\text{C}$  or  $5^{\circ}\text{C}$  there is no appreciable loss, even when stored over periods of several months. When wet and kept at room temperature ( $24$  to  $27^{\circ}\text{C}$ ) *P. pestis* toxin slowly loses potency, and the rate of loss appears to be greatest in the first few days of storage. At  $37^{\circ}\text{C}$  there is no loss of potency after 1 hour, but at  $56^{\circ}\text{C}$  or higher the

toxin rapidly becomes inactivated, and at  $65^{\circ}\text{C}$  for 30 minutes is almost completely inactive. The crude preparations were found to behave in a similar fashion at these temperatures; this agrees with findings of others working with crude fractions (Meyer, 1949). Lyophilized purified toxin retains its toxicity and antigenicity for at least six months when kept at room temperature.

The mouse toxicity of purified toxin is not affected at the pH range of 4.0 to 10.0. Purified toxin was kept at room temperature for 1 hour in the following buffers: acetate, 0.0140 M,

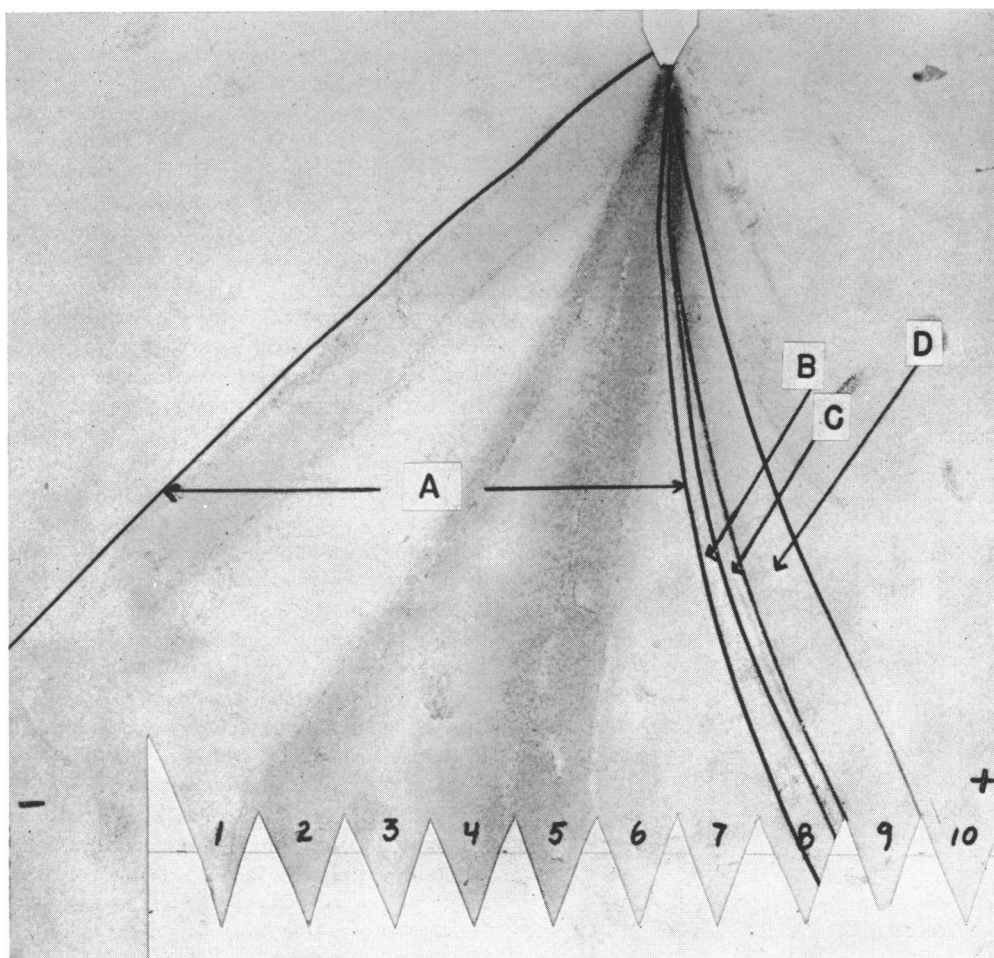


Figure 8. Paper electrophoretic pattern of *Pasteurella pestis* toxin hydrolyzate demonstrating location of unknown components.

A = identified amino acids; B\* = fluorescent component; C\* = absorbing component; D = unknown ninhydrin reacting component.

Paper: no. 3 mm Whatman; current: 3 MA; voltage: 150 v; electrolyte: 1 N acetic acid, pH 2.4.

\* Unknown components B and C were marked prior to ninhydrin treatment.

pH 4.0; phosphate, 0.0098 M, pH 6.0; phosphate, 0.0400 M, pH 8.0; and borate 0.0500 M, pH 10.0. No significant change in toxicity was noted. In another experiment toxin was stored in phosphate buffer, pH 8.0, at 24 to 27 C and titrated in mice after intervals of 7, 14, and 28 days (table 3). It was found that the loss of potency of toxin in phosphate buffer at room temperature was not as appreciable as that observed in distilled water. Whereas in 28 days at room temperature the amount required for one LD<sub>50</sub> increased from 9 to 56  $\mu$ g when kept in

water, it only changed from 9 to 25  $\mu$ g when stored in the buffer.

#### DISCUSSION

The methods described for the purification of *P. pestis* toxin yield a product which appears to be highly homogeneous. However, the toxin may still contain contaminating proteins which have not yet been detected. Additional criteria, such as constancy of composition and quantitative serological reactions, are still required. On the other hand, the available electrophoretic and

TABLE 3

The  $LD_{50}$  of purified and crude *Pasteurella pestis* toxin at various temperatures and time intervals

Temperature	Toxin	Diluent	Interval		
			7 days	14 days	28 days
C -70	Purified* Lot no. 8	Distilled water	—	13	13
			16	16	13
4 24-27			41	43	56
24-27	Purified† Lot no. 8	Phos- phate buffer, pH 8.0	18	18	25
24-27	Purified* Lot no. 8	Distilled water	0 min	30 min	60 min
			9	—	—
			—	—	10
			—	75	—
			—	>320	—
37			—	>320	—
56			—	—	—
65			—	—	—
80			—	—	—
24-27	Crude‡ Lot no. A	Distilled water	10	—	—
37			—	—	11
56			—	36	—
80			—	160	—

\* Concentration—640  $\mu$ g/ml.

† Concentration—1 mg/ml.

‡ Crude, ammonium sulfate precipitated toxin; concentration—5.0 mg/ml.

serologic data suggest that *P. pestis* toxin prepared in the manner described is of considerable purity and provides a valuable reagent for serologic and pharmacological investigations.

The chemical scheme presently employed for fractionation can undoubtedly be modified. However, the procedures were adopted as it became evident that each one resulted in either an increase in toxicity per unit of dry weight or a decrease of envelope antigen or other contaminants, as nucleoprotein. It should be emphasized that although purified *P. pestis* toxin requires more weight of toxin to kill than most crude preparations, this does not necessarily indicate the presence of nontoxic impurities but rather that some inactive toxoid may have

been formed during purification. This is substantiated by the finding that purified toxins of low potency nevertheless contain as much specific antigenicity as do more toxic preparations.

Because of the considerable trauma to the toxin molecule involved in the above method we have recently attempted to use only paper electrophoresis for fractionating crude extracts. Crude dialyzed sodium chloride extracts are precipitated with  $(NH_4)_2SO_4$ , the precipitate dissolved in water, dialyzed, and freeze-dried. The dried crude toxin is then dissolved in buffer and fed into the paper electrophoresis cell and the toxin collected at the proper drip points. By successively repeating the electrophoretic separation 2 or 3 times, purified toxin can be obtained. The stained pattern on the filter paper serves as a guide to the number of runs necessary. Although this method eliminates many chemical steps of the original procedure, the small yields obtainable in the paper cell result in approximately the same over-all manufacture time, and the total yields of toxin are no higher.

Approximation of the amount of toxin in the intact plague bacillus has been attempted, and in the experiment tabulated in table 1 the percentage of toxin has been estimated to be 3.5 per cent.<sup>2</sup> It must be emphasized that this is undoubtedly low, and recent studies in this laboratory indicate that *P. pestis* contains at least 50 per cent more toxin demonstrable as serologically active material than by toxicity tests in the mouse. The details of these experiments are described in another publication (Warren *et al.*, 1955).

*P. pestis* toxin is a protein of rather small

<sup>2</sup> This figure was arrived at from the data shown in table 1 in the following manner: The percentage loss can be calculated from the total units of toxin at the beginning and at the end of the procedure as follows:  $268,610:30,000/100\%:X$ ; where  $X$  = total yield of toxin and  $100 - X$  = % loss of toxin. In this case  $X = 11\%$ . Thus 89% of toxin is lost during the purification process. In terms of dry weight 30,000 units of toxin represent an 11% yield. To calculate the total amount of the relatively pure toxin in 268,610 units of crude material, the following ratio can be used:  $0.078:11\%/X:100$ ; where  $X$  = amount of toxin in the crude preparation. In this case  $X = 0.70$  g. Since 0.70 g of pure toxin is present in 20 g of acetone dried bacilli, the percentage of toxin in these cells is 3.5.

molecular weight; on the basis of sedimentation and diffusion data the molecular weight of the toxin is of the order of 74,000. (In calculating the molecular weight, the partial specific volume has been assumed to be 0.75.)

The products of hydrolytic breakdown of the material are of unusual interest. Although 12 naturally occurring amino acids are always separable, the presence of unknown components absorbing and fluorescing in the ultraviolet is of fundamental interest. It is tempting to speculate that the presence of these unknown substances is in some way related to toxicity.

#### SUMMARY

By means of various chemical procedures and continuous paper electrophoresis, a toxin has been isolated from *Pasteurella pestis* in a state of considerable purity. The LD<sub>50</sub> of the purest preparation is 1 µg or 222 LD<sub>50</sub> per kilo of mouse per mg of toxin nitrogen when injected intravenously and 5 µg or 44 LD<sub>50</sub> per kilo of mouse per mg of toxin nitrogen by the intraperitoneal route. The toxin contains 14 per cent nitrogen, 1.9 per cent sulfur, and not more than 0.2 per cent phosphorus. It is free of nucleoproteins, carbohydrate, and capsular material. It behaves as a homogenous protein in the ultracentrifuge and electrophoresis cell, having a molecular weight of the order of 74,000. The purified toxin is antigenic and gives rise to a specific antitoxin when injected into rabbits.

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