ANTIGENS OF *BORDETELLA PERTUSSIS*

II. Purification of Heat-Labile Toxin[1]

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ABSTRACT

Banerjea, A. (Rocky Mountain Laboratory, Hamilton, Mont.) and J. Munoz. Antigens of *Bordetella pertussis*. II, Purification of heat-labile toxin. J. Bacteriol. **84**:269–274. 1962.—A mild method of separating heat-labile toxin of *Bordetella pertussis* from other cellular components is described; it consists of absorbing toxin on a diethylaminoethyl cellulose column and eluting it with a gradient concentration of NaCl. Toxin preparations thus obtained consisted mainly of protein; their toxicity was destroyed by trypsin but not by ribonuclease or deoxyribonuclease.

The heat-labile toxin of *Bordetella pertussis* was first demonstrated by Bordet and Gengou (1909). This substance is lethal and dermonecrotic for rabbits, guinea pigs, and mice (North et al., 1939; Strean and Grant, 1940; Wood, 1940). The pathological changes produced in some animals resemble those observed in children dying from whooping cough, and thus it has been thought that this toxin plays an important role in the pathogenesis of the disease (Dominici, 1907; Bordet and Gengou, 1909; Gallavan and Goodpasture, 1937; Sprunt, Martin, and McDearman, 1938). Local and systemic reactions observed after vaccination with pertussis vaccine have been suspected to be due to this toxin (Felton and Verwey, 1955).

Its extreme lability has been a great obstacle to purification of the toxin, which is easily destroyed by heat, alcohol, phenol, formalin, toluene, and other agents, as well as by filtration through Chamberland, Berkefeld, or Seitz filters (Bordet and Gengou, 1909; Teissier et al., 1929· Miller, 1934; Evans and Maitland, 1937; Wood, 1940; Pennell and Thiele, 1951; Pittman, 1952).

The toxic activity has been separated by immunological means from the so-called agglutinogen, precipitinogen, and complement-fixing antigens (Evans and Maitland, 1937; Verwey and Thiele, 1949), thus demonstrating that the toxin is distinct from other antigenic components of the cell. The toxin has been partially purified by isoelectric precipitation (Strean and Grant, 1940; Flosdorf and Kimball, 1940), and by methanol precipitation under specified conditions (Robbins and Pillemer, 1950). Many alcohols, including methanol, have deleterious effects on the toxin, and it seems more advantageous to employ other methods of purification. Cellulose column chromatography has been recently used with some degree of success (Billaudelle et al., 1960).

This paper presents results obtained in attempts to establish the chemical nature of purified preparations of the heat-labile toxin from *B. pertussis*.

MATERIALS AND METHODS

Cells. *B. pertussis* strains 2926 and 2927 (obtained from Merck Sharp and Dohme, West Point, Pa.) were used.

Preparation of protoplasm. The procedure for preparing protoplasm has been previously described (Munoz, Ribi, and Larson, 1959). Cells were ruptured by treatment in a Mickle disintegrator, centrifugation at 29,000 $\times$ *g* for 1 hr, and lyophilization of the supernatant fluid containing toxin. This material (protoplasm) was used as starting material for isolation of heat-labile toxin.

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Ion-exchange chromatography. Diethylaminoethyl (DEAE) cellulose columns were prepared in a Pyrex glass tube (1 cm diam and 23 cm long). The end of the tube was constricted into a capillary encased in a glass tube, 4.5 cm long, which fitted into a drop counter.

Dried protoplasm was dissolved either in distilled water or in appropriate buffer and adsorbed onto the cellulose column. The toxin was eluted by a gradient of from 0 to 1 M NaCl in 0.015 M phosphate buffer.

Toxicity tests. Different amounts of protoplasm or chromatographic fractions were injected intraperitoneally into Swiss albino mice in a volume of 0.5 ml or less. Protoplasm and toxic fractions killed mice within 24 to 48 hr. Results were recorded 48 hr after injection of the preparations.

Colorimetric estimations. Protein content of samples was estimated by a slight modification of the procedure of Lowry et al. (1951), using Folin-Ciocalteau reagent (Folin and Ciocalteau, 1927). A standard curve prepared with egg albumin was used as a reference for quantitative estimations.

The carbohydrate content of samples was estimated by a modification of the method of Thomas (Glick, 1955), employing H_2SO_4 and tryptophan. Quantitative estimations were done by referring to a standard curve made with known amounts of glucose.

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were estimated by making a hydrolyzate according to the method of Morse and Carter (1949) and determining the amount of pentose by the orcinol reaction, to estimate RNA (Brown, 1946), and determining deoxypentose by the diphenylamine reaction, to estimate DNA (Glick, 1955). Standard curves for RNA and DNA were used for quantitative estimations of these substances.

Spectrophotometric readings. Spectrophotometric readings were taken in a Beckman DU spectrophotometer between wavelengths of 220 and 300 mμ. Optical density values were plotted against wavelengths.

Treatment with trypsin. To 1.6 ml of each fraction, or sample to be treated, was added 0.2 ml of a solution of trypsin containing 250 μg per ml of 0.015 M phosphate buffer (pH 7.1). The mixture was kept at room temperature for 1 hr; then 0.5-ml portions were injected intraperitoneally into each mouse.

The enzyme was found to be active under the above conditions when tested by a modification of the method of Kunitz (1947) for the determination of tryptic activity on casein.

Treatment with ribonuclease. To 1.6 ml of each fraction or sample was added 0.2 ml of a solution of ribonuclease containing 125 μg per ml of 0.015 M phosphate buffer (pH 7.1). The mixture was allowed to stand at room temperature for 1 hr, and was then tested, as in the case of trypsin treatment, for its toxicity in mice. The enzyme was found to be active when tested by a slight modification of the method described by McDonald (1955).

Treatment with deoxyribonuclease. The method used was similar to that employed for ribonuclease, except that enough $MgSO_4 \cdot 7H_2O$ was added to the reaction mixture to give a final concentration of 0.005 M. The enzyme was found to be active under the conditions of the experiment, when tested as described by McDonald (1955).

RESULTS

Toxicity and chemical composition of protoplasm. A preparation of *B. pertussis* protoplasm, made as described above, was titrated for its toxicity in mice. Of five mice injected with as little as 0.01 ml per mouse, two died; five of five receiving 0.05 ml died. This protoplasm was lyophilized and tested for toxicity on a weight basis; 20 μg of the dried material dissolved in saline was sufficient to kill all the mice injected (Table 1).

An aqueous solution of protoplasm containing 200 μg per ml gave optimal ultraviolet-light absorption at a wavelength of 260 mμ (Fig. 1). By the method of Warburg and Christian (1941), the amount of protein in a protein-nucleic acid mixture was estimated from spectrophotometric

TABLE 1. *Toxicity of protoplasm after lyophilization*

Amount of dried protoplasm per mouse	Toxicity*
μg	
500	5/5
200	4/5
100	9/10
50	3/3
40	4/5
20	8/8
10	1/3

* Deaths per total number injected.

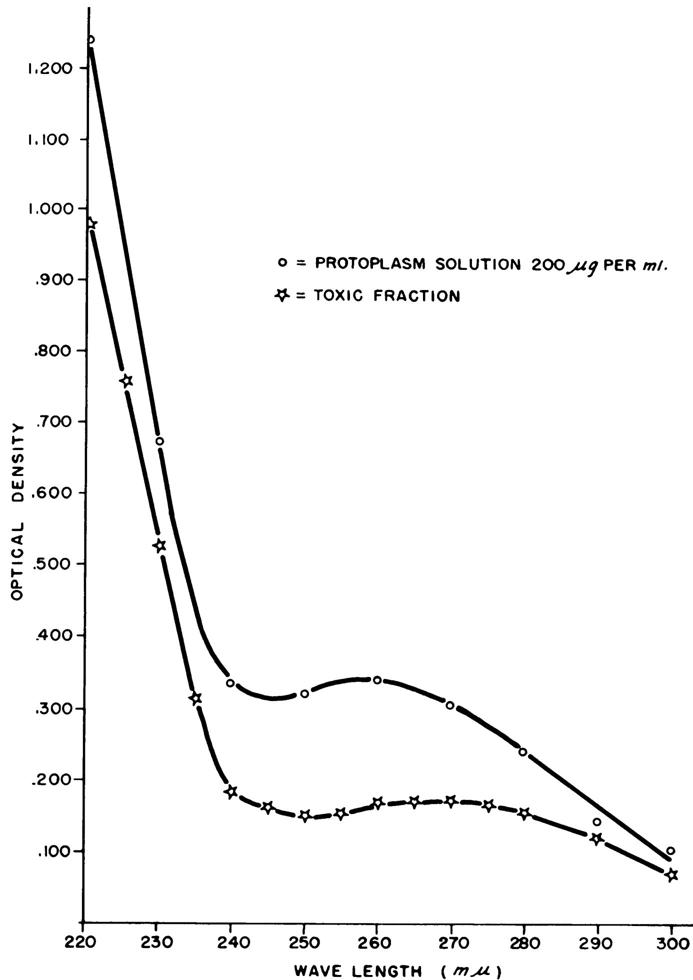

FIG. 1. *Spectrophotometric readings of protoplasm and purified heat-labile toxin from Bordetella pertussis*

readings. The aqueous solution of protoplasm containing 200 μg per ml was calculated to contain approximately 117 μg of protein and about 20 μg of nucleic acid. The remaining 63 μg in the protoplasm presumably consisted of carbohydrates and other materials. Chemical estimations confirmed the spectrophotometric findings. Thus, 200 μg of protoplasm contained 124 μg of protein, 9 μg of RNA, and 4 μg of DNA; 23 μg of carbohydrate were also found.

Absorption and elution of heat-labile toxin from DEAE cellulose columns. Cellulose columns were prepared by the method of Sober and Peterson (1954) and Peterson and Sober (1956). DEAE cellulose columns from 5.5 to 6 cm were used. Dry protoplasm (5 mg) dissolved in 7.5 ml of 0.015 M phosphate buffer (pH 7.1) was passed through the column; the column was washed four times with 7-ml amounts of phosphate buffer, and then the elution process was started. A continuous sodium chloride gradient (from 0 to 1 M sodium chloride) in 0.015 M phosphate buffer (pH 7.1) was applied. About 5 ml (120 drops) per fraction were collected and tested for toxicity in mice. The toxin was completely absorbed onto DEAE columns and was not removed by buffer (Table 2). The first two fractions collected after the sodium chloride gradient had been applied had no toxin present. Toxin was detected in the third fraction, and most of the toxicity was found in fractions 4, 5, and 6. The ultraviolet-absorbing property of fractions 4, 5, and 6 could not be

determined because of the low concentration of materials in these fractions. A small peak, however, was detected at 270 mμ and, in one case, at 280 mμ, which indicated a shift from the nucleic acid-type peak obtained with whole protoplasm to a protein-type absorption peak. By employing DEAE cellulose in a batch method of absorption, it was possible to obtain somewhat more concentrated preparations, which gave more reliable absorption data. The absorption obtained with one of these preparations is shown in Fig. 1, where a shift of absorption toward that of a typical protein is clearly shown.

Effect of enzymes on toxicity of heat-labile toxin of B. pertussis. The above experiments gave an indication that the heat-labile toxin of *B. pertussis* might be protein in nature. To obtain stronger evidence regarding the nature of this toxin, the effect of trypsin, deoxyribonuclease, and ribonuclease on the toxicity of various preparations was studied. Protoplasm and samples of various toxic preparations obtained from DEAE cellulose columns were submitted to the action of these enzymes, and then tested for their toxicity in mice. As controls for the toxicity of enzymes, 0.2 ml of a solution containing a concentration similar to that used to digest the sample was also injected into mice. In all cases, trypsin completely destroyed the toxic effect of whole protoplasm or toxic fraction but ribonuclease and deoxyribonuclease did not (Tables 3 and 4). These results strongly indicate that the heat-labile toxin of *B. pertussis* is a protein or a complex molecule containing protein as one of its main components.

TABLE 2. *Combined toxicity test of fractions obtained from DEAE cellulose columns*

Fraction*	Toxicity†
First eluate	0/15
Washings with buffer	
I	0/15
II	0/15
III	0/15
IV	0/15
Fractions with NaCl gradient	
1	0/15
2	0/15
3	3/15
4	12/15
5	14/15
6	13/15
7	9/15
8	3/9
9	0/9
10	0/9

* Fraction (0.5 ml) was injected intraperitoneally into each mouse.

† Deaths per total number of mice injected.

TABLE 3. *Effect of trypsin, deoxyribonuclease, and ribonuclease on toxicity of whole protoplasm of Bordetella pertussis**

Protoplasm	Enzyme	Toxicity†
μg/0.5 ml		
100	—	8/9
100	Trypsin	0/9
—	Trypsin control	0/3
100	Deoxyribonuclease	8/9
—	Deoxyribonuclease control	0/3
100	Ribonuclease	8/9
—	Ribonuclease control	0/3

* Each mouse received (intraperitoneally) 0.5 ml of test material containing 100 μg of protoplasm and either 14 μg of trypsin or 7 μg of deoxyribonuclease or ribonuclease. Controls received either protoplasm alone or enzyme alone in the same volume.

† Deaths per total number of mice injected.

TABLE 4. *Effect of trypsin, deoxyribonuclease, and ribonuclease on toxic fractions obtained from a DEAE cellulose column eluted with an NaCl gradient**

Toxic fraction†	Enzyme	Toxicity‡
4	—	2/3
4	Trypsin	0/3
4	Deoxyribonuclease	2/3
4	Ribonuclease	2/3
5	—	3/3
5	Trypsin	0/3
5	Deoxyribonuclease	2/3
5	Ribonuclease	2/3
—	Trypsin	0/3
—	Deoxyribonuclease	0/3
—	Ribonuclease	0/3

* Each mouse received (intraperitoneally) 0.5 ml of the test material containing the toxin and either 14 μg of trypsin or 7 μg of deoxyribonuclease or ribonuclease. The controls received the same volume of either toxic fraction alone or enzyme alone.

† See Table 2.

‡ Deaths per total number of mice injected.

DISCUSSION

Ion-exchange cellulose columns have provided an effective method of purifying a variety of proteins and nucleoproteins from animal, plant, and microbiological sources (Sober and Peterson, 1958). DEAE cellulose columns were found effective in adsorbing the *B. pertussis* heat-labile toxin; subsequent elution of toxin was accomplished with a gradient of NaCl in 0.015 M phosphate buffer (pH 7.1). Billuadelle et al. (1960) also separated the heat-labile toxin from other cellular components by DEAE column chromatography. Their starting material had an MLD of 10 μg for a 10-g mouse, and their purified material had an MLD of 20 to 40 μg. This failure to reduce the MLD on attempted purification of the toxin may reflect the instability of this substance. Our starting material, after lyophilization, was lethal to mice (16 to 18 g) in amounts from 10 to 20 μg.

By assuming that the toxin is a simple protein and that all the protein in our starting material was toxin, it can be calculated that the lethal dose for mice is about 6 μg. This dose, however, would be much lower in highly purified toxin preparations. Owing to the instability of the substance, the exact lethal dose on a weight basis could not be determined with any degree of accuracy.

Spectrophotometric and chemical analysis showed that purified toxic fractions contained mainly protein, and traces of RNA, DNA, and carbohydrate. The absence of RNA, DNA, and carbohydrate in some purified toxic fractions indicated that the toxin, as believed by Strean and Grant (1940), is composed mainly of protein (see also Billaudelle et al., 1960). Results obtained with enzymatic degradation strongly support this view, since the proteolytic enzyme, trypsin, completely destroyed the toxicity of toxic preparations. The heat-labile toxin has been thought by some (Yamamoto et al., 1952, 1953) to be a nucleoprotein. No evidence to support this claim was found.

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