# ISOLATION OF PROTECTIVE ANTIGEN FROM BORDETELLA PERTUSSIS

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No satisfactory purification of the protective antigen from Bordetella pertussis has ever been accomplished. The protective antigen appears to be located on the cell wall (Billaudelle et al., 1960; Munoz, Ribi and Larsen, 1959; Yoshida et al., 1955). Treating the organisms with sodium deoxycholate, Barta (1963) obtained a protective antigen with feeble toxicity. Pusztai, Joo and Juhasz (1961) reported that the higher is the protective activity of a pertussis vaccine, the higher is its histamine sensitizing activity. A stroma-adsorbed protective antigen (Pillemer, Blum and Lepow, 1954) contained the histamine sensitizing activity (Maitland, Kohn and MacDonald, 1955). Zone electrophoresis failed to separate histamine sensitizing activity from protective activity in a crude extract of the acetone-dried organisms. From this result, Munoz (1963) postulated that a single entity was responsible for both the activities.

The present report deals with isolation of two fractions of protective antigen from the supernatant of the sonicated organisms mainly by centrifugation in sucrose density gradient and with some properties of the two fractions, the one with $S_{20,w}$ of 22 and the one showing membraneous structure.

## Results and Discussion

B. pertussis phase I, strain Tohama, grown on Cohen-Wheeler's agar plates at 37 C for 48 hr was suspended in 0.05 M borate buffer, pH 9.0, at a concentration of 1,500 International Opacity Units/ml. The suspension was sonicated at 10 kc for 40 min at cold and centrifuged at 20,000 xg for

30 min. A 0.2-ml portion of the supernatant containing 5.2 mg protein was placed on top of 4.8 ml of 20-55% sucrose density gradient in 0.1 M borate buffer, pH 9.0. This was spun in a SW 39 rotor in a Spinco ultracentrifuge model $L_2$ at 39,000 rev/min for 15 hr. As shown in Fig. 1, the material was resolved into 3 protein peaks. A portion from each peak was heated at 50 C for 20 min and this was injected into mice to test for protective activity by the International method (World Health Organization, 1964).

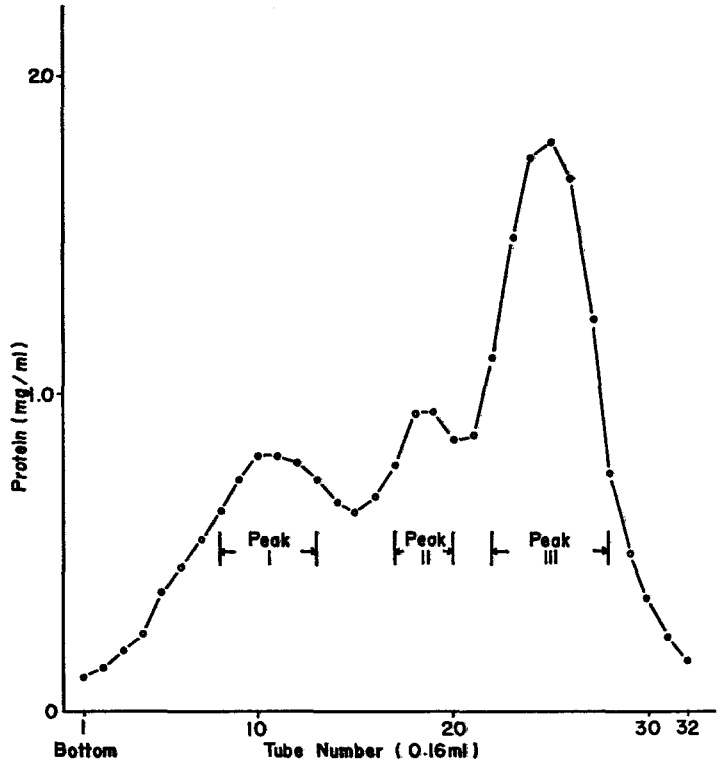


Fig. 1 Sucrose density gradient centrifugation of the supernatant of the sonicated organisms. Protein content was determined with Folin reagent and expressed as serum albumin equivalent.

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As seen in Fig. 2, zone electrophoresis resolved peak I into the ribonucleoprotein fraction (tubes no. 35-40) and the protein fraction (tubes no. 21-27). The protein fraction was subjected to centrifugation in 20-55% sucrose density gradient, which gave a fraction of protective antigen,

referred to as GZ fraction, at the same relative position as peak I of the pattern in Fig. 1.

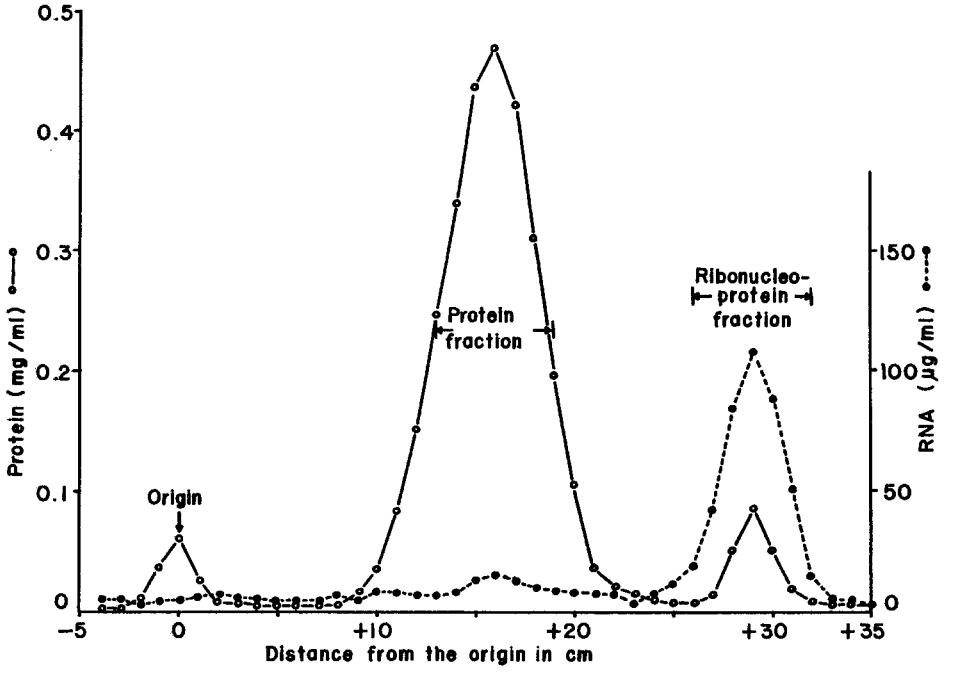


Fig. 2 Zone electrophoresis of peak I fraction (in Fig. 1). Supporting medium, Geon 427-S; buffer, 0.05 M borate (pH 9.0); a current, 2 mA/cm$^2$ for 12 hr; extraction, each segment extracted with 6 ml of the buffer; protein determination, the same as described in the legend to Fig. 1; ribonucleic acid determination, by the orcinol test.

Peak II (Fig. 1) was also subjected to zone electrophoresis and the protein fraction migrating toward the anode was removed. Centrifugation of this fraction (3.9 mg protein/0.2 ml) in 5-20% sucrose density gradient for 4 hr yielded a protein peak in tubes no. 9-13 as in Fig. 3. This fraction was found to possess a $S_{20,w}$ of 22.0.

Table 1 summarizes protective activity of this and GZ fraction, which possessed a similar level of the activity. No protective activity was demonstrated in peak III (Fig. 1) nor in the ribonucleoprotein fraction (Fig. 2).

Ultracentrifugal analysis and electrophoresis on a cyanogum plate strongly indicated homogeneity of the 22S antigen fraction. A single band in agar gel diffusion test was observed against an anti-phase I whole cell rabbit serum. Chemical analyses revealed that the 22S antigen contains

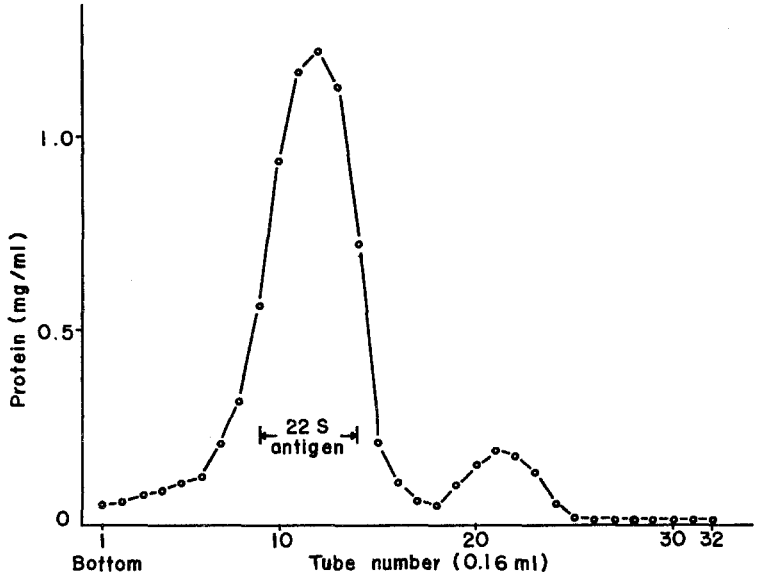


Fig. 3 Sucrose density gradient centrifugation of protein fraction (in Fig. 2). Protein determination, the same as described in the legend to Fig. 1.

protein in 76%, lipid in 23%, and carbohydrate inclusive of amino sugar in 2%. No deoxyribonucleic acid nor ribonucleic acid was detected. Electron microscopic observation of negatively stained preparations demonstrated that the antigen consists of cylindrical molecules (120 x 120 A) and that each molecule is composed of four layers of ring-shaped substructures each consisting of several particles arranged in a pattern like flower petals (Fig. 4). It was also shown that the antigen molecules are located inside the cell wall or on the cell membrane (Fig. 5).

Most of the histamine sensitizing activity contained in the supernatant

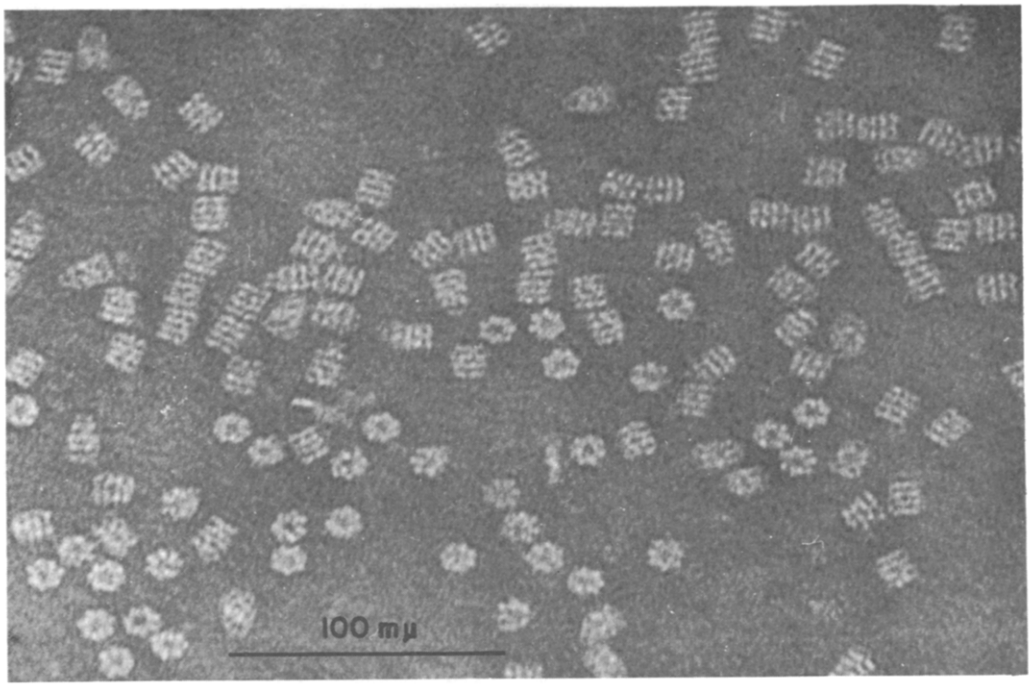


Fig. 4 Electron micrograph showing the molecules of the 22S antigen negatively stained with 1% phosphotungustic acid.

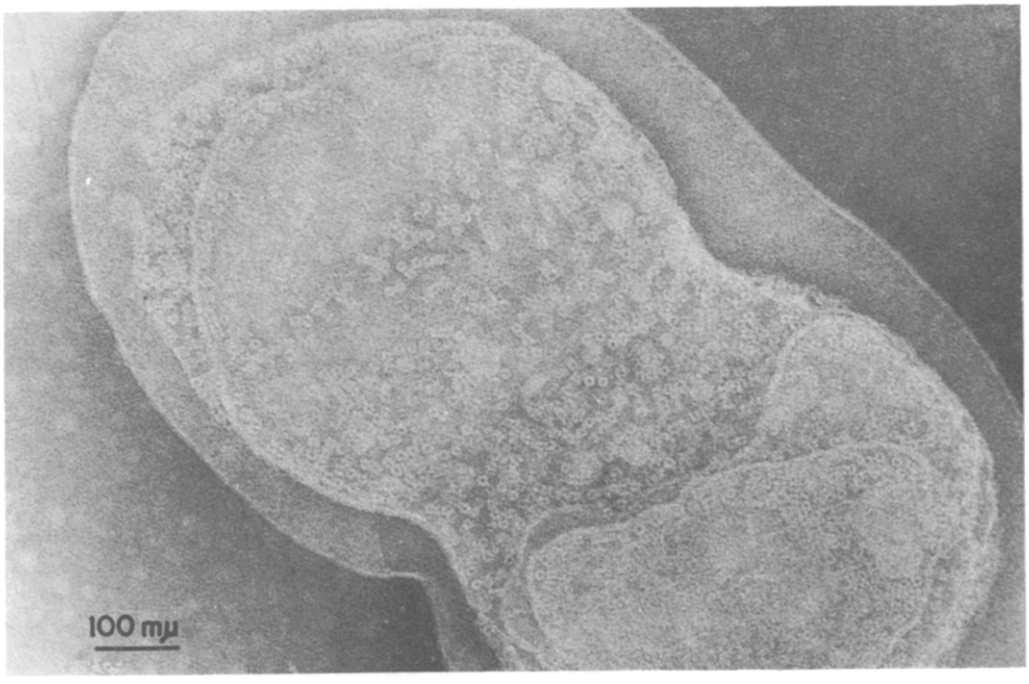


Fig. 5 Electron micrograph showing a cell after treatment with trypsin. Cells suspended in 0.05 M phosphate buffer, pH 8.0, at 10 International Opacity Units/ml were incubated with 0.1% trypsin at 37 C for 20 min and negatively stained with 1% phosphotungustic acid.

Table 1

Protective potency of GZ fraction and the 22S antigen in comparison with the starting material

| Fraction | Exp. I | | | Exp. II | | | Exp. III | | |
|---|---|---|---|---|---|---|---|---|---|
| | Dose (μg) | S/T* | $ED_{50}$ (μg) | Dose (μg) | S/T | $ED_{50}$ (μg) | Dose (μg) | S/T | $ED_{50}$ (μg) |
| Supernatant of sonicated organisms | 200 | 14/16 | 44.8 (23-87)** | 200 | 15/16 | 29.0 (16-53) | 120 | 13/16 | 30.1 (16-58) |
| | 40 | 6/16 | | 40 | 9/16 | | 24 | 7/16 | |
| | 8 | 3/16 | | 8 | 3/16 | | 4.8 | 2/16 | |
| GZ fraction | 200 | 15/16 | 21.0 (15-37) | 40 | 14/16 | 12.6 (7-24) | 120 | 15/16 | 11.8 (6-24) |
| | 40 | 12/16 | | 8 | 4/16 | | 24 | 10/16 | |
| | 8 | 3/16 | | 1.6 | 3/16 | | 4.8 | 5/16 | |
| 22S antigen | 200 | 15/16 | 22.8 (12-44) | 40 | 14/16 | 7.2 (4-14) | 120 | 15/16 | 9.4 (5-19) |
| | 40 | 10/16 | | 8 | 8/16 | | 24 | 12/16 | |
| | 8 | 4/16 | | 1.6 | 3/16 | | 4.8 | 5/16 | |

* S/T indicates the ratio of number of mice survived to that tested.
** Figures in parentheses represent the fiducial limits at 95% level of probability.

of the sonicate ($HSD_{50}$=10-23 μg) was recovered in fraction III (Fig. 1). No significant activity was demonstrated in the 22S antigen fraction ($HSD_{50}$ > 450 μg).

Analyses of GZ fraction determined protein in 60%, lipid in 24%, carbohydrate inclusive of amino sugar in 8% and ribonucleic acid in 1%. Electron microscopic observation of the fraction revealed membraneous structure and association of some 22S antigen molecules with it. It has not been clarified as yet whether the protective activity possessed by GZ fraction is due to the membrane itself or to the 22S antigen associated with the membrane.

The 22S antigen dissociated into smaller subunits with $S_{20,w}$ of about 3 when treated with 6 M urea or 1% sodium deoxycholate, or at alkalinity above pH 10. At least 5 distinct bands were demonstrated when such

dissociated preparations were subjected to disc electrophoresis. It has remained to be solved, however, whether or not all the components with different mobilities possess the protective activity.

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