

STUDIES ON THE ULTRASTRUCTURE OF BORDETELLA  
PERTUSSIS

I. MORPHOLOGY, ORIGIN, AND BIOLOGICAL ACTIVITY OF STRUCTURES PRESENT  
IN THE EXTRACELLULAR FLUID OF LIQUID CULTURES OF  
BORDETELLA PERTUSSIS\*

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The biological effects which follow the injection into animals of *Bordetella pertussis* cells or their products are diverse and include such reactions as: increased susceptibility to histamine, serotonin, active and passive anaphylaxis and cold stress; enhancement of experimental allergic encephalomyelitis; production of homocytotropic antibodies; an adjuvant effect on conventional antibody production; and hyperleukocytosis with a predominant lymphocytosis (reviewed in reference 1).

Recently, Morse and Bray (2) have shown that histamine-sensitizing activity (HSA) and lymphocytosis-promoting factor(s) (LPF) were found primarily in the extracellular fluid of liquid cultures rather than in association with cells. Both activities were essentially confined to a fraction insoluble in buffers of low ionic strength. Because of difficulties in maintaining solubility, as well as a tendency for the active material to aggregate at high concentrations and to adsorb onto a variety of substances, further characterization was difficult to achieve.

In an attempt to gain relevant information of a different type, culture supernatant fluids were examined, after negative staining, by high resolution electron microscopy. Two distinctive particles were revealed and their morphogenesis, properties, and possible role in biological reactions comprise the substance of this report.

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### *Materials and Methods*

*Organisms.*—*Bordetella pertussis* strain 3779B is a phase I strain kindly supplied by Dr. J. M. McGuire of Eli Lilly and Co., Indianapolis, Ind. It is the strain which has been extensively used in previous studies on pertussis-induced lymphocytosis. Other *Bordetella* strains employed were: *B. pertussis* 51755, 6229, and 18398, all phase I; *B. pertussis* L51 and L54, both designated phase IV; *B. parapertussis* E113 and 147/5; and *B. bronchiseptica* 22-067 and 8752. We are very grateful to Dr. Jean Dolby of the Lister Institute of Preventive Medicine, Elstree, Herts., England, for supplying these strains and for her helpful advice.

*Escherichia coli* K12 C600 was kindly supplied by Dr. T. Naha of the National Institute for Medical Research, London and *E. coli* 9106 by Dr. M. T. Parker of the Colindale Public Health Laboratory, London.

*Media and Growth Conditions.*—The liquid and solid media employed for culturing phase I *B. pertussis* were essentially unchanged from that previously described. Hedley-Wright solid and liquid media were also used. Incubation was at 36.5°C.

*Animals.*—Male Balb/c mice from the National Institute for Medical Research were used when they were 6-8 months of age. Leucocyte counts of tail blood samples were performed by conventional hemocytometer methods.

*Electron Microscopic Methods.*—Specimens for negative staining were applied as a drop to grids which were covered with parlodion film stabilized with carbon. The drop was allowed to dry partially. Then a drop of 2% sodium dodecatungstosilicate in distilled water was applied and the excess removed after 20-30 sec.

Specimens for thin sectioning were prepared by the Kellenberger technique (3) followed by dehydration in graded alcohols and embedding in araldite. The thin sections were stained with lead citrate. All specimens were examined in a Siemens I Elmiskop.

### RESULTS

#### *Elements Found in the Extracellular Fluid of Phase I B. pertussis.*—

Liquid media was inoculated with organisms from a 3 day agar culture of strain 3779B. The initial concentration of cells was  $6-10 \times 10^7$ /ml. After incubation in shallow layers for 3-5 days, maximum density of  $2-4 \times 10^{10}$  cells/ml was achieved. Thimerosal was added to a final concentration of 0.02%; the particulate resin was removed by filtration of the culture through fine gauze; and the organisms were sedimented by centrifugation. The supernatant fluid was then examined directly after negative staining.

As can be seen in Fig. 1, two distinctive elements were present, in addition to round particles of heterogeneous size. One of these measured 40-110  $m\mu$  in length and was characterized by one or two terminal or subterminal prominences. The bulbous portions averaged 35  $m\mu$  in diameter, whereas in the narrower portions the width was 12-13  $m\mu$ . A common appearance was that seen in Fig. 2. With the presence of two bulbous ends, these particles had the conformity of dumbbells. It was of note that the negative stain only infrequently penetrated into the dumbbell particles.

The second distinctive particle was filamentous with a diameter of approximately 20 A. These narrow filaments ranged in length from 40-70  $m\mu$  (Figs. 1, 3).

Neither particle was found in uninoculated culture media treated in the

same fashion, indicating that they were not artifacts induced in the medium during incubation.

*Electron Microscopy of Phase I B. pertussis Cultures at Various Times during Growth.*—

Aliquots from fluid cultures of strain 3779B were removed at various time periods after inoculation. A sample of the whole culture aliquot was retained and the remainder was centrifuged. The cell-free supernatant fluid was removed and the bacterial pellet was resuspended to initial volume in 0.05 M tris(hydroxymethyl)aminomethane(Tris)-0.5 M NaCl, pH 7.4. This buffer was chosen to avoid aggregation of any cell-associated particles. The specimens were then stained with silicotungstate and examined in the electron microscope.

During the first 48 hr of culture small numbers of both elements were found free in the extracellular fluid. However, during this interval filaments were found attached to the cells (Fig. 4). In addition, projections averaging 12–15  $\mu$  in width and of varying lengths were present. As seen in Figs. 4 and 5, these were sometimes several hundred millimicrons long, had bulbous termini, constrictions at regular intervals, and were often penetrated by the stain.

During the subsequent 48 hr of culture fewer filaments and projections were found attached to the cells while a greater concentration of free filaments and dumbbells was present in the supernatant fluid. After 4 days of culture virtually no cell-associated appendages were seen other than those clearly adherent to, rather than part of, the cell surface.

Examination of suspensions of agar-grown organisms at various time periods showed a sequence of changes similar to those seen in liquid cultures.

*Studies of Other Bordetella Species and E. coli.*—

The three other strains of phase I *B. pertussis* examined showed similar morphological entities free in the extracellular fluid, or attached to cells depending upon the time of sampling. Phase I *B. pertussis* strains can only be grown when blood, albumin, charcoal, starch, or some other absorbent is present, and in the medium used in these studies an anion exchange resin, Dowex 1, was employed. Since the resin might have bound substances affecting the structure of the bacterial cells, it was important to determine whether the morphological entities were related to this particular medium. It was first shown that soluble starch could be substituted for the resin and that the same morphological appearances were noted.

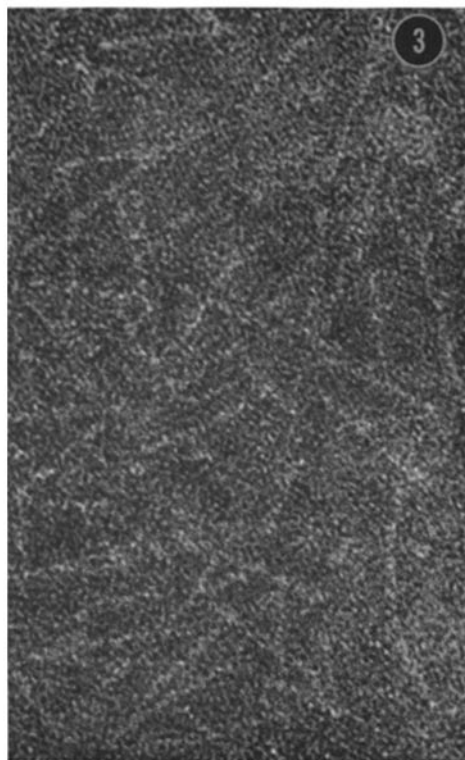
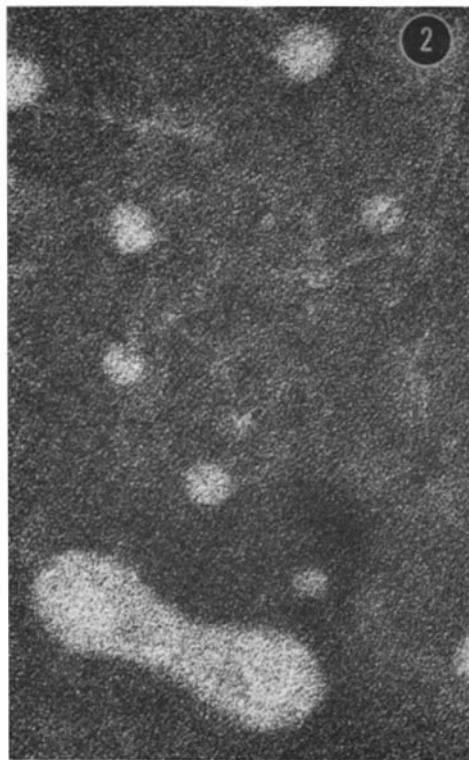
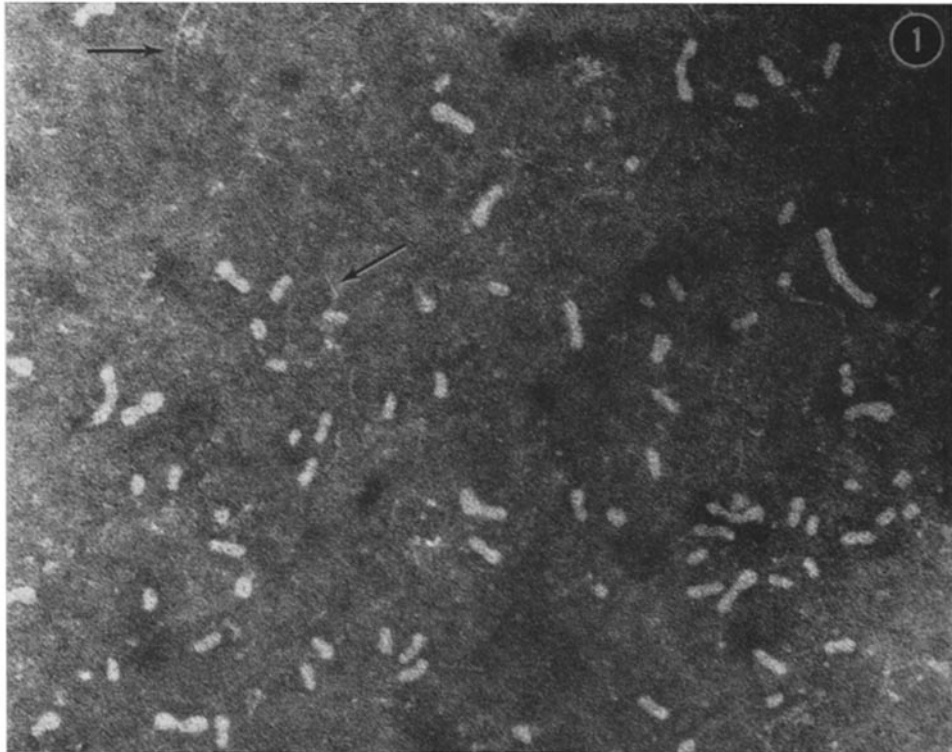
Other *Bordetella* species, as well as phase IV strains of *B. pertussis*, grow well in conventional nutrient media. Strains of *B. parapertussis* and *B. bronchiseptica*

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FIG. 1. Negatively stained supernatant fluid of *B. pertussis* culture; 4 day growth in liquid medium. Numerous dumbbell shaped particles as well as fine filaments (→) are present.  $\times 140,000$ .

FIG. 2. Higher magnification of dumbbell shaped particle. Round particles of varying diameters are also present.  $\times 400,000$ .

FIG. 3. Fine filaments at higher magnification.  $\times 400,000$



were therefore cultured in both ordinary nutrient medium (Hedley-Wright) and in the medium designed for phase I *B. pertussis*. Most of the observations

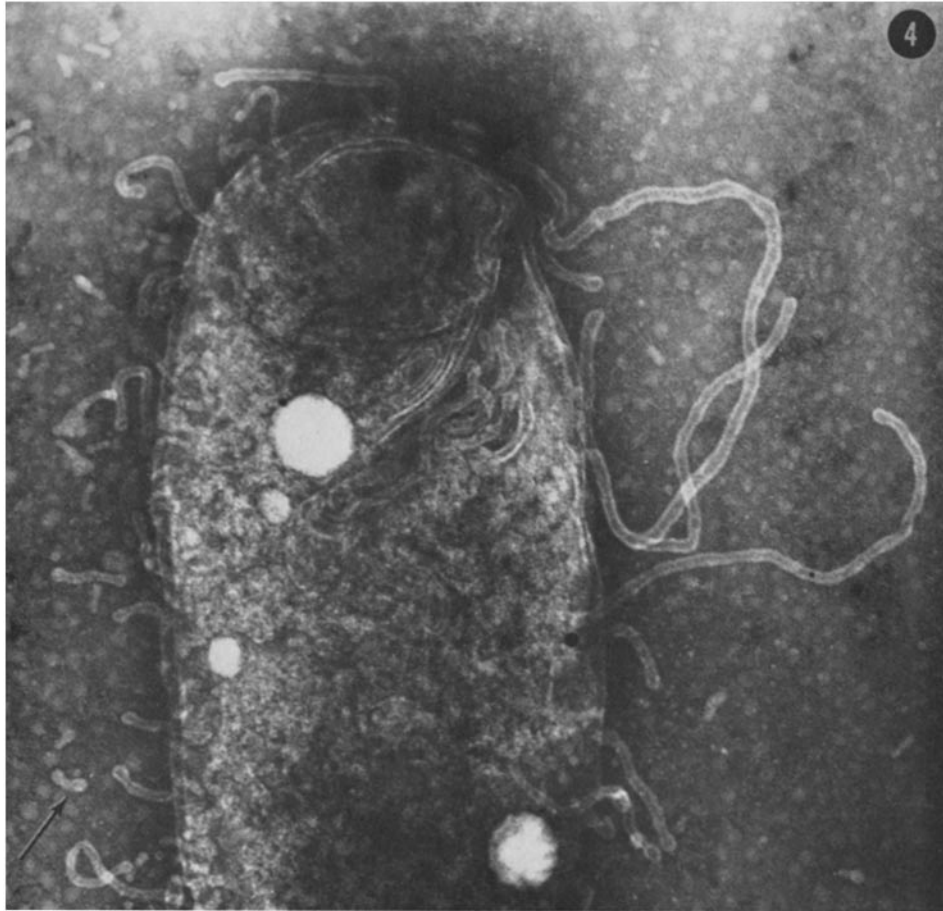


FIG. 4. Negatively stained *B. pertussis* cell cultured for 2 days in liquid medium. Tentacular projections with bulbous termini and constrictions at intervals are present. Extracellular dumbbell shaped particles, believed to have been derived from the projections, are also present (→). Salt crystals are apparent over the bacterium and in the background.  $\times 100,000$ .

were made on cultures during the latter part of log phase growth. Numerous free filaments and dumbbells of identical morphology to those seen in cultures of *B. pertussis* in phase I were found. In addition, filaments attached to the bacterial cell as well as the projections noted in *B. pertussis* cells were seen

during the early stages of growth. There was no difference in morphological appearance of cells or extracellular elements when samples prepared from both media were compared.

Phase IV strains of *B. pertussis* were distinctive in that only a few filaments were observed, whereas both the dumbbells and projections were apparent. Although no complete survey of genera was undertaken, two strains of *E. coli*

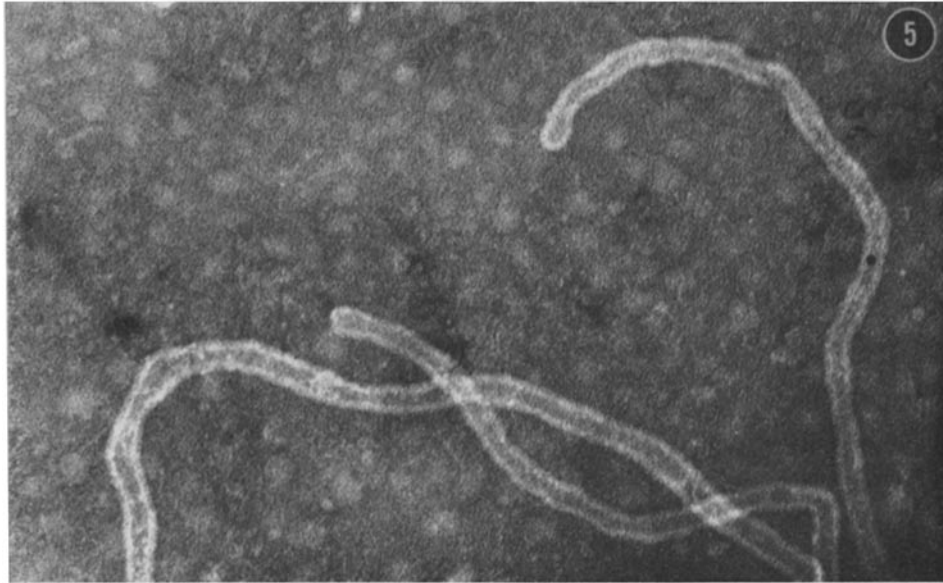


FIG. 5. Cellular projections.  $\times 200,000$

were examined. No filaments were seen, but projections from the cell were observed as seen in Fig. 6. *E. coli* K12 C600 is piliated (fimbriated) and it is clear from Fig. 6 that the pili have a quite different appearance from both the filaments and cell projections.

*Origin of the Extracellular Elements Found in B. pertussis Cultures.—*

From the sequence of events found in growing cultures of phase I *B. pertussis*, it seemed likely that the extracellular dumbbells were derived from the cell projections found earlier in culture. The dimensions, constrictions, various sizes, and bulbous termini all favored the notion that these projections fragmented from distally inward, resulting in the appearance of the extracellular elements. However, it was not clear from the available evidence from what cell site the projections arose. Similarly, the origin of the filaments was unknown. Experiments were performed to help ascertain the relationship of these structures to known bacterial components.

After 24 hr of growth in liquid media, ampicillin (Penbritin, Beecham Research Laboratories, Brentford, England) was added to cultures of *B. pertussis*

3779B to achieve a final concentration of 2  $\mu\text{g}/\text{ml}$ . After an additional 24 hr of incubation the cultures were examined under the electron microscope.

As this was a bactericidal concentration of the drug, most of the cells were too disorganized to yield any substantive information. In those that remained intact a striking finding was that the ends of the projections were markedly

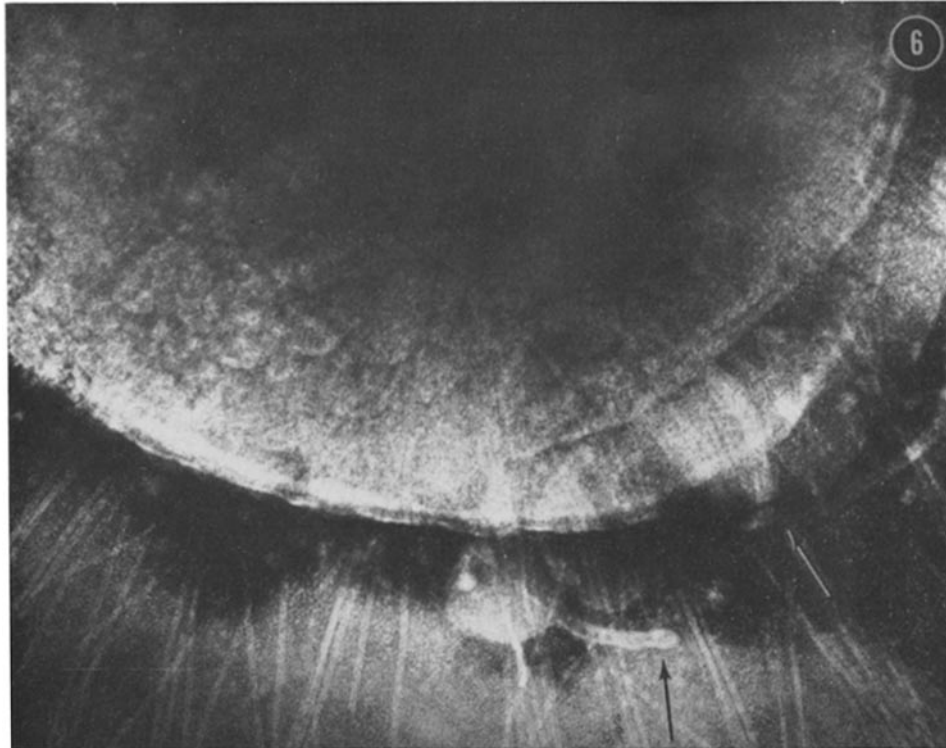


FIG. 6. Piliated *E. coli* with surface projections ( $\rightarrow$ ).  $\times 140,000$

enlarged (Fig. 7). Moreover, the bulbous portions of the free dumbbells were also enlarged, giving credence to the notion that they were indeed derived from the projections.

Spheroplasts of *B. pertussis* have been produced in the presence of glycine (4, 5) and therefore such forms were examined. They were produced by culturing organisms for 48 hr in the usual phase I medium containing 1% of glycine. During the time period of growth the optical density at 530  $m\mu$  increased approximately 10-fold. As seen in Fig. 8, the protoplast was retracted away from the cell wall giving a "fried egg" appearance. It is readily apparent that both the projections and filaments were attached to the cell wall, suggest-

ing that they were not anchored to deeper layers of the cell. Further support to this thesis was derived from our finding of both structures on fragments of cell walls, devoid of internal constituents, which were occasionally seen (Fig. 9).

In an attempt to gain more information on the structure and origin of the particles, organisms grown for 48 hr were centrifuged, fixed without washing,



FIG. 7. *B. pertussis* cell incubated in the presence of ampicillin. Projections have dilated termini. An internal membranous structure is visible.  $\times 140,000$ .

stained, and examined after thin sectioning. As shown in Figs. 10 and 11 the cell walls were frequently ruffled and projections were evident. The projections were clearly bordered by two electron-opaque layers separated by a less dense layer—that is, a trilaminar unit membrane structure. Similarly, the extracellular particles were also membranous. The appearance of the projections and extracellular particles in thin sections suggested that they were the same as the projections and dumbbells found after negative staining.

In common with other Gram-negative cells, the cell wall of *B. pertussis* is



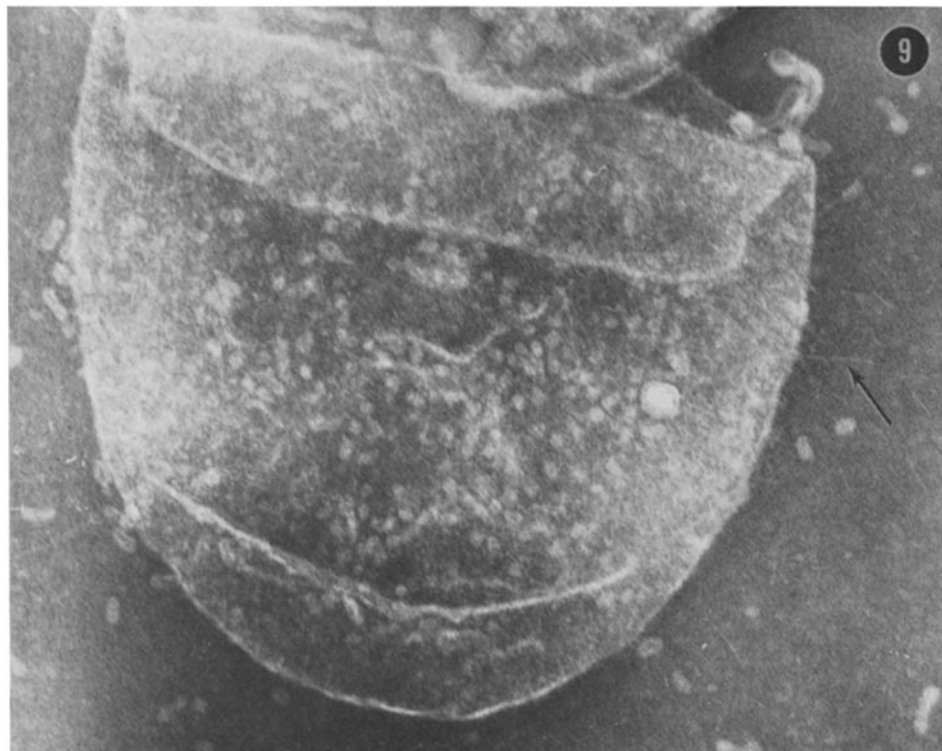
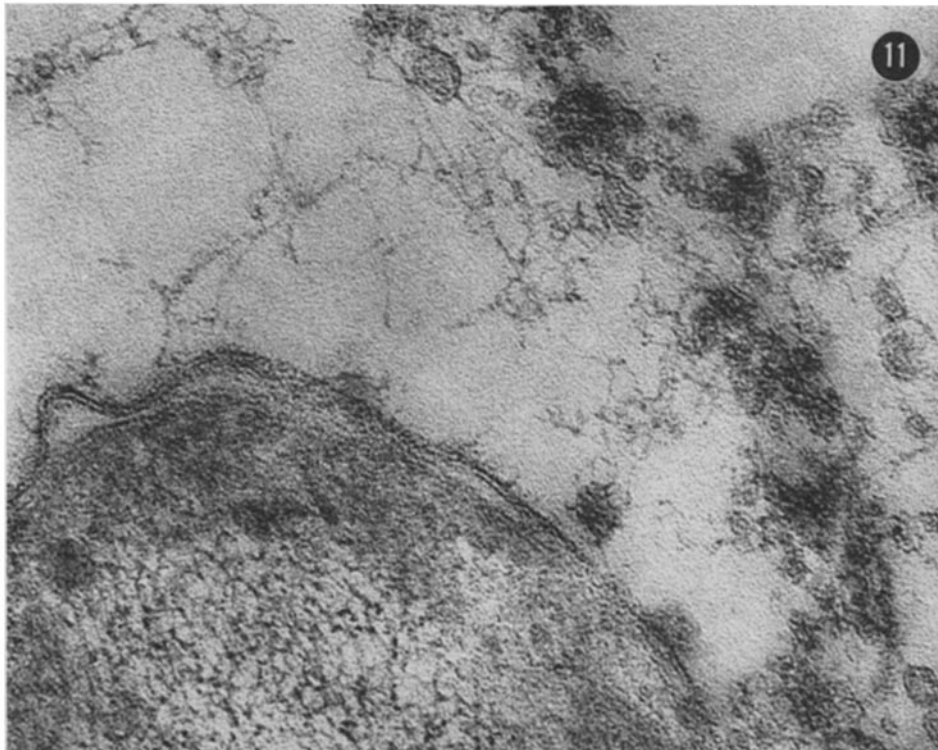
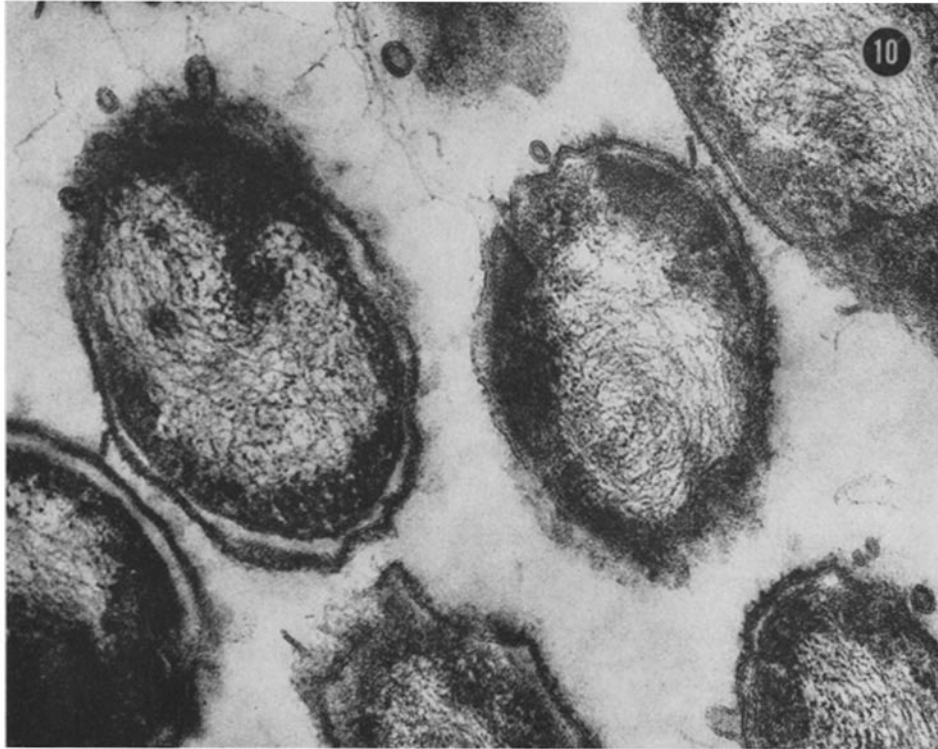


FIG. 8. Glycine-induced spheroplast of *B. pertussis*. The protoplast is retracted from the cell wall and both the surface projections and fine filaments (→) appear to originate in the wall.  $\times 140,000$ .

FIG. 9. Cell wall fragment of *B. pertussis* to which both projections and filaments (→) are attached.  $\times 100,000$ .



FIGS. 10 and 11. Thin sections of *B. pertussis* cells grown for 48 hr in liquid medium. Both surface projections and extracellular particles have a trilaminar unit membrane structure.  $\times 100,000$  and  $200,000$ .

composed of five layers. The outer three layers, "outer membrane" or "outer track," together have the appearance of a unit membrane. The outer track is separated from a deeper, dense layer by a less dense layer. The innermost dense layer represents the mucopeptide. As is the case with many other organisms, the mucopeptide layer of *B. pertussis* cell walls is poorly demarcated and not always readily apparent. Review of numerous sections showed no evidence for the association of an inner dense layer with either the surface projections or the extracellular material. As noted above, however, this possibility could not be firmly ruled out.

Filaments were not identified with certainty in the thin sections.

*Properties of the Extracellular Elements.*—

Both filaments and dumbbells were decreased in number after passage of *B. pertussis* supernatant fluids through Millipore filters with an average pore size of 0.45  $\mu$  (Millipore Filter Corporation, Bedford, Mass.). After dialysis of supernatant fluids against 0.05 M Tris-0.5 M NaCl, pH 7.4, both elements appeared to be present to approximately the same extent as in undialyzed preparations. After dialysis against 0.05 M Tris at the same pH or against distilled water, the supernatant fluids became turbid as had been noted previously. After centrifugation at 8000 g for 30 min, the resulting supernatant fluid was found to have a marked decrease in the two particles under discussion. Examination of the pellets resuspended in buffer containing 0.5–1.0 M NaCl revealed what appeared to be matted dumbbells and filaments, but morphological detail was obscured by the gross character of the aggregates.

When the supernatant fluid was centrifuged at 100,000 g for 18 hr, both elements were decreased in the 100,000 g supernatant and appeared to be present in the pellet which also proved difficult to disperse adequately.

Incubation of thimerosal-free culture supernatant fluids at 37°C for 60 min with 1 mg/ml of trypsin, pronase, lysozyme (muramidase), ribonuclease, or deoxyribonuclease, had no effect on the morphology of the particles. Culture supernatant fluids were also incubated in the presence of 0.05 M Na metaperiodate, pH 7.4, at room temperature for 2 hr. The reaction was then terminated by the addition of excess glucose. No effect on the morphology of either particle was noted. It is of interest that under these conditions periodate does not affect LPF (unpublished observations).

Sodium dodecyl sulfate, final concentration 0.5%, was added to 2-day cultures and after 15–30 min at room temperature the mixture was examined. Although the conformation of the bacterial cells remained unchanged, the outer surface was now composed of granular small particles; the projections were no longer present. In addition, similar particles were present extracellularly but the dumbbells were absent and the filaments were matted.

Absorption of supernatant fluids with fowl or sheep erythrocytes or sheep erythrocyte membranes resulted in a marked decrease in the number of both extracellular elements.

*Gradient Centrifugation of B. pertussis Supernatants.*—As noted above, a variety of physical and chemical procedures did not provide any useful method for distinguishing between the two distinctive extracellular particles present in

cultures of phase I *B. pertussis*. This was of central interest because the behavior of both particles in the procedures used paralleled that of LPF. For example, LPF was only moderately affected by prolonged exposure to high concentrations of proteolytic enzymes for prolonged periods of time, did not pass through a 0.45  $\mu$  filter, and was destroyed by sodium dodecyl sulfate (2). In addition, both LPF and HSA aggregated in solutions of low ionic strength, and in the reports of others HSA, like LPF, has been shown to be relatively resistant to proteolytic enzymes (6).

However, in view of their marked morphologic differences, it seemed possible

TABLE I  
*Distribution of Lymphocytosis Stimulating Activity Following Centrifugation of B. pertussis Culture Supernatants in CsCl Density Gradients*

Fractions (top to bottom)	Density	Filaments	Dumbbells	Stimulation of lymphocytosis
3 hr* 1	1.09	++++	±	++++
2	1.18	+++	++	++
3	1.26	±	++++	+
4	1.34	0	±	0
5	1.41	0	0	0
18 hr* 1	1.15	±	0	0
2	1.19	++++	±	++++
3	1.26	+	++	+
4	1.34	0	++++	0
5	1.38	0	0	0

\* Time of centrifugation at 100,000 *g* 4°C.

that the two elements might be separated on the basis of their density. Accordingly, gradient centrifugation in CsCl solutions were performed in the following manner.

CsCl solutions were made up in 0.005 M Tris-0.02 M MgCl, pH 7.8. On top of 0.8 ml of CsCl,  $\rho = 1.5$ ; 1.0 ml of CsCl of densities of 1.30, 1.25, and 1.20 were added successively. 1.0 ml of culture supernatant fluid was then added and the tubes were centrifuged at 100,000 *g*, 4°C, for various time periods in the SW39 rotor of the Model L ultracentrifuge (Spinco Division, Beckman Instruments, Inc., Fullerton, Calif.). Four 1.0 ml fractions were removed from the top, while the bottom fraction contained 0.8 ml. The samples were then dialyzed against 0.05 M Tris-0.5 M NaCl, pH 7.4, and injected intravenously in equivalent doses into mice. Leukocyte counts were determined 3 days later. Electron microscopy of the fractions was also performed.

As can be seen in Table I, lymphocytosis was induced only in those fractions in which filaments were located, and the degree of lymphocytosis was related to their concentration. Recovery of activity was 80-90%. In preliminary

experiments, it has also been found that histamine-sensitizing activity was also greatest in fractions rich in filaments.

#### DISCUSSION

Electron microscopic examination of supernatant fluids of *B. pertussis* cultures has revealed the presence of two distinctive morphologic elements. The first of these, termed "dumbbells" in this report, were shown, by sequential examination of growing cultures, to derive from extensions of the surface structure of the organism. Thus, early in culture, few particles of this type were found free in the extracellular fluid, but tentacular projections, of similar width, were present on the organism. These projections often had constrictions at intervals approximating the length of the extracellular particles found later in the course of bacterial growth. At this later period processes on the bacterial cells were no longer found. The processes clearly originated from the cell wall and appeared to be composed of the outer two layers of the wall ("outer track" or "outer membrane") but the presence of mucopeptide could not be excluded. On negative staining, the stain penetrated the core of the processes, but only infrequently did it penetrate the extracellular particles. On thin sections the external boundary of both was triple layered with the morphologic characteristic of a unit membrane.

The presence of the processes is in accord with the model of the cell wall of Gram-negative bacteria postulated by Bayer and Anderson (7). Unit membrane structure has also been shown in endotoxic lipopolysaccharide from *Veillonella alcalescens* and it was thought that the endotoxin derived from the outer membrane of the cell wall (8, 9). Moreover, membranous blebs are found on the surface of lysine-requiring *E. coli* cultured under lysine-limiting conditions, and extracellular globular and plate-like material can be found extracellularly (10). These particles are more rounded than the membranous particles described here and have much greater variation in diameter. They are believed to represent extracellular lipoglycopeptide released by the breaking off of the blebs which form from the outer layer of the cell wall under the conditions of lysine deprivation.

In the studies reported herein, it would appear that as a normal feature of the growth of *B. pertussis*, the outer layer of the cell wall grows in an unbalanced fashion, and then by an unknown mechanism, is fragmented. On morphologic grounds, there is no reason to believe that this is an agonal phenomenon.

The question of whether the extracellular membrane units are, or contain, endotoxin has not been resolved. It was of interest, however, that after phenol-water extraction of samples rich in these membranes, residual membranes were not found in the aqueous phase. However, this could have been a quantitative matter since it is not known to what extent losses of material might occur in the procedure used. Also against the possibility that the membranes were rich

in lipopolysaccharide was the finding that only small amounts of carbohydrate and less than 1% lipid were present in a fraction which, on the basis of current studies, must have been quite rich in membranes (2). Direct testing of the relationship between the membranous particles and endotoxin is required.

Although these studies were mainly concerned with phase I *B. pertussis*, processes and extracellular particles of similar morphology were found in two other *Bordetella* species as well as in phase IV strains of *B. pertussis*. Although not as prominent, the processes were also found in two strains of *E. coli*. Thus, it is possible that the sequence of events observed is a general phenomenon in Gram-negative bacteria.

Lane has recently suggested that the mouse-lethal toxin of *B. pertussis* is released from the cell via the formation of vesicles which pinch off from the cell wall (11). These "vesicles" may be identical with the membranous elements described here, which can stick to the surface of the organism, or they may represent residual processes. However, as Lane has emphasized, there is thus far no direct evidence that toxin is released through defined structural components.

A second particle present in supernatant fluids of phase I *B. pertussis* cultures was filamentous and measured only 20 A in diameter. It too seemed to derive from the cell wall but precise morphogenetic detail was difficult to achieve. Filaments of similar appearance have not, to our knowledge, been recorded as elements of bacterial structure. (However, Dr. C. W. Forsberg has kindly shown us electron micrographs of a marine pseudomonad in which similar filaments seem to be present [12].) The filaments do not have the morphological characteristics for pili (fimbriae) and differ markedly from the filaments derived from the cell walls of mycobacteria (13, 14). Although the filaments could be a type of pilus, it would seem proper to denote them with the more general term of "filament" until a function can be assigned to them or until further analyses reveal their chemical nature. The possibility that they are filamentous bacteriophages has not been excluded (15, 16).

As in the case of the membranous particles, the filaments were found free in the extracellular fluid at the time at which maximum growth had been reached, and few were cell associated.

Filaments were found in cultures of *B. bronchiseptica* and *B. parapertussis*, but have not been identified with certainty in the case of phase IV strains of *B. pertussis* or in *E. coli* strains.

Despite their disparate morphologic appearance, the filaments and membranous particles had many similarities. The similar features included: (a) inability to pass through a 0.45  $\mu$  filter; (b) aggregation in media which was not hypertonic; (c) absorption to erythrocytes and erythrocyte membranes; and (d) insusceptibility to proteolytic enzymes, muramidase, nucleases, and periodate under the conditions employed.

However, the particles could be separated on the basis of their densities by gradient centrifugation in CsCl. It was found that induction of lymphocytosis was associated only with those gradient fractions in which filaments were found, and the degree of lymphocytosis paralleled the concentration of filaments in the preparation. It was of note that the distribution of histamine-sensitizing activity followed the same pattern.

It should be emphasized that these results do not necessarily prove that the filaments have biological activity. The high resolving power of the electron microscopic technique employed permits the demonstration of material of molecular dimensions as well as the structural elements observed. Thus, although the filaments and membranous particles were separated from each other, other extracellular particles, presumably representing excreted proteins of varying sizes and densities, were also present in the gradient fractions. If the filaments of phase I *B. pertussis* strains are responsible for lymphocytosis they must differ from those found in cultures of *B. parapertussis* and *B. bronchiseptica* since the latter two species do not have lymphocytosis-promoting activity.

#### SUMMARY

Two distinct particles have been recognized in the extracellular fluid of *B. pertussis* cultures. Both appeared to arise from the surface (cell wall) of the organism.

One of these, a membranous particle, seemed to derive from long projections on the organism composed of the outer membrane of the cell wall.

The second particle, a fine filament, was not readily comparable with any previously described bacterial structure.

The two particles could be separated from each other by gradient centrifugation in CsCl.

Lymphocytosis-promoting factor and histamine-sensitizing activity were only associated with fractions containing the fine filaments.

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