# BIOLOGICAL ACTIVITIES IN EXTRACTS OF *PASTEURELLA PESTIS* AND THEIR RELATION TO THE "pH 6 ANTIGEN"

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## ABSTRACT

BICHOWSKY-SLOMNICKI, LEAH (The Weizmann Institute of Science, Rehovoth, Israel) AND SHLOMO BEN-EFRAIM. Biological activities in extracts of *Pasteurella pestis* and their relation to the "pH 6 antigen." J. Bacteriol. **86**:101–111. 1963.—Three kinds of biological activities could be found in crude extracts prepared from all the tested strains of *Pasteurella pestis*: cytotoxicity for monocytes, ability to agglutinate red blood cells, and induction of primary inflammatory reactions of the skin. All these biological activities were related to the presence of the "pH 6 antigen." In addition, fractions of the pH 6 antigen devoid of agglutinating activity against red blood cells, but possessing the other biological activities, could be isolated. The active biological principles appear to be protein in nature. The possible role of the described biological activities in the pathogenesis of plague is discussed.

It has previously been found that incubation of *Pasteurella pestis* in a xylose-Casamino Acids medium at a pH below 6.7 and a temperature above 35 C caused the formation of an antigenic surface component, called "pH 6 antigen." Further experiments in vivo showed that the same component was synthesized by *P. pestis* cells in rabbits and mice and that the rate of mortality was increased in groups of mice inoculated with virulent cells containing the pH 6 antigen (Ben-Efraim, Aronson, and Bichowsky-Slomnicki, 1961). In view of the in vivo findings, it has been suggested that the pH 6 antigen may be involved in the pathogenesis of plague. To obtain further information on this point, different biological activities of crude extracts, as well as of purified preparations, obtained from cells with pH 6 antigen, were determined.

## MATERIALS AND METHODS

*Strains.* The avirulent strain TRU was used as the prototype for the preparation of crude extracts. This strain is able to synthesize the pH 6 antigen (Ben-Efraim et al., 1961) and lacks some of the known antigenic fractions of *P. pestis* (Crumpton and Davies, 1956). Other strains of *P. pestis* used were the avirulent strains TS, TSR, and 14, and the virulent strains MP6, M23, Kimberley, and Alexander. Strain 14 was kindly supplied by D. A. L. Davies, Porton, England, and strain Alexander was obtained from the Communicable Disease Center, U.S. Public Health Service. The other strains were obtained from sources described in an earlier communication (Ben-Efraim et al., 1961).

*Culture and incubation conditions.* The conditions for growth of the bacteria, and for their subsequent incubation, were identical with those described previously (Aronson and Bichowsky-Slomnicki, 1960).

*Preparation of crude extracts. P. pestis* cells were harvested with a Sharples centrifuge and resuspended in a neutral solution of 1 M potassium thiocyanate (Amies, 1951), with a final concentration of 6 g of packed cells per 100 ml of solution. The suspension was shaken on a reciprocating shaker at room temperature for 6 hr. The clear supernatant fluid was separated by centrifugation at 20,000 × *g* and dialyzed in the cold against distilled water. The protein in the dialyzed portion was precipitated by addition of solid ammonium sulfate up to 100% saturation. The precipitate was dissolved in 0.05 M phosphate buffer (pH 8.0) and dialyzed against the same buffer at 4 C. This solution was designated "crude extract."

*Ammonium sulfate fractionation.* The crude extract was subjected to fractional precipitation at 4 C by increasing amounts of neutral saturated ammonium sulfate solution, ranging from 0 to

70% saturation. The different fractions were stirred during the salt addition and allowed to precipitate for approximately 30 min. The precipitates were collected by centrifugation at 10,000 rev/min, dissolved in 0.05 M phosphate buffer (pH 8.0), and dialyzed against the same buffer in the cold (4 C).

*Protein estimations.* The protein concentration was determined by the method of Lowry et al. (1951).

*Paper electrophoresis.* The electrophoresis was carried out on cellulose acetate strips (Oxo Ltd., London), on a Kohn Universal electrophoresis apparatus (Shandon Co. Ltd., London), according to the procedure described by Kohn (1958). Amido black and Ponceau S were used for staining.

*Preparative electrophoresis.* The procedure for preparative electrophoresis was essentially the same as described by Robbins and Lipmann (1958). Geon 426 (polyvinyl chloride particles; B. F. Goodrich Co., Akron, Ohio) was used. The electrophoretic separation was carried out for about 18 hr at 4 C with 105 v. The proteins were eluted from the block sections by 0.05 M phosphate buffer (pH 8).

*Calcium phosphate gel adsorption.* For adsorption, the gel suspension reagent (lot 518-240-2 16, 4% solids; Sigma Chemical Co., St. Louis, Mo.) was centrifuged (2,500 $\times$ *g* for 5 min) and the supernatant liquid discarded. The packed gel was mixed thoroughly with the protein solution in a ratio of 8.4 mg of gel solids per mg of protein. The gel-protein suspension was allowed to stand for 15 min, and the gel was removed by centrifugation. The gel containing the adsorbed protein was washed with distilled water and eluted with 10-ml portions of increasing concentrations (0.01 to 0.2 M) of phosphate buffer (pH 6.8). In some of the experiments, the elution of the adsorbed protein was continued with 0.5 and 1.0 M phosphate buffer (pH 6.8), then with 0.01 M Veronal buffer (pH 9.0) and 0.01 M sodium hydroxide glycine buffer (pH 10.1), and finally with 0.05 M sodium hydroxide. All operations were carried out at room temperature.

*Sedimentation tests.* Sedimentation tests were carried out on a Spinco model E ultracentrifuge at 21° with a Schlieren optical system. Runs were made in a 4°, 12-mm cell at a rotor speed of 56,100 rev/min and an optical angle of 50 or 55°.

*Experimental animals.* Rabbits (2 to 2.5 kg) and guinea pigs (400 to 500 g) were used.

*Preparation of immune sera.* Immune sera were prepared in rabbits by two methods: (i) by injection of living cells of strain TRU containing the pH 6 antigen, and (ii) by injection of the crude extract of the same cells. Adsorbed immune sera, which contained antibodies only against the pH 6 antigen, were also prepared. The schedules for immunization and the procedure used for adsorption were described previously (Ben-Efraim et al., 1961).

*Immunochemical tests.* Gel diffusion tests were conducted by a technique of double diffusion on agar plates (Ouchterlony, 1953), under the same conditions as described in a previous paper (Ben-Efraim et al., 1961). Immunoelectrophoresis was performed on a Kohn Universal electrophoresis apparatus as described by Grunbaum and Dong (1962).

*Agglutination of red blood cells.* Sheep red blood cells were used, if not otherwise stated. The red blood cell suspensions and isotonic buffer solutions were prepared as described by Borduas and Grabar (1953). The fractions to be tested were diluted with isotonic buffered saline at pH 6.4, up to a total volume of 1.2 ml, and mixed with 0.5 ml of a 2.5% red blood cell suspension. The suspension was allowed to stand for 15 min at room temperature, and the red blood cells were then collected by light centrifugation. The presence of cell aggregates was noted after resuspension of the red blood cells in 0.5 ml of isotonic buffered saline (pH 6.4).

*Preparations of monocyte suspensions from guinea pigs.* Peritoneal monocytes were harvested from guinea pigs 4 days after an intraperitoneal injection of 20 ml of 2% starch solution in physiological saline. They were suspended in Hanks solution (Hanks and Wallace, 1949) containing 3% homologous serum and distributed in test tubes in 1-ml quantities of approximately $10^6$ cells per ml. The test tubes were kept at 37 C for 2 hr. The liquid was then withdrawn and replaced by new Hanks medium containing 30% normal guinea pig serum and 5 $\mu$g of streptomycin per ml. The fractions to be tested were added to such maintenance cultures of monocytes in serial dilutions in quantities of 0.1 ml per test tube.

*Preparation of monocyte suspensions from rabbits.* The technique of harvesting and maintaining rabbit monocytes was as described by Fong, Schneider, and Elberg (1957). The monocytes were distributed in Leighton tubes in 2-ml

quantities at a concentration of $2.5 \times 10^5$ cells per ml. The fractions were introduced into the Leighton tubes together with the monocyte suspension in serial dilutions and in quantities ranging from 0.02 to 0.4 ml. The monocyte suspensions were incubated at 37 C for 3 days and were examined microscopically at daily intervals, starting 3 hr after the beginning of incubation.

*Skin tests.* White depilated rabbits were used. The material tested was injected intradermally in 0.2-ml quantities.

## Results

*Antigenic components in crude pH 6 extracts.* It was found by gel diffusion tests that crude extracts prepared from cells of strain TRU, incubated at 37 C and pH 6, contained the pH 6 antigen and at least three additional antigenic fractions. The line of the pH 6 antigen present in the crude extracts was identical with the precipitation line given by this antigen attached to intact TRU cells (Fig. 1). Crude extracts prepared from TRU cells incubated at 37 C and pH 7 (Fig. 1), 28 C and pH 6, and 28 C and pH 7.0, respectively, did not contain the pH 6 antigen. These extracts contained the other antigenic fractions detected in the pH 6 crude extracts. The same results were obtained with crude extracts of strains TS, TSR, 14, Kimberley, Alexander, and MP6 and its mutant M23.

*Biological activities.* The following biological activities could be detected in the crude pH 6 extracts of the above-mentioned strains: cytotoxicity for monocytes, ability to agglutinate red blood cells, and induction of a primary inflammatory reaction of the skin. These biological activities were absent in extracts of cells incubated at a neutral pH or at 28 C.

*Cytotoxic activity.* The crude pH 6 extracts of *P. pestis* cells caused a degeneration of monocytes under in vitro conditions. The first effect was observed 3 hr after the addition of the extract and was characterized by aggregation of the monocytes. Later on, granulation appeared inside the cells, followed by distortion of the monocyte layer and complete degeneration (Fig. 2). Destruction of the monocytes occurred even when the maintenance medium containing the crude pH 6 extract was replaced by fresh medium after 30 min of incubation. The unit of cytotoxic activity was arbitrarily defined as the smallest quantity of protein ($\mu$g) that caused a marked reaction during 24 hr of incubation. The specific activity was expressed as units of cytotoxic activity per mg of protein. The specific activity of crude pH 6 extracts ranged from 3 to 8 units per mg of protein. Extracts prepared from cells incubated at 37 C and pH 7, 28 C and pH 7, or 28 C and pH 6 were devoid of cytotoxic activity, even when preparations containing much higher concentrations of protein (up to 2.5 mg) were used. Similar results were obtained in the case of guinea pig and rabbit monocytes.

Immune rabbit sera prepared against TRU cells incubated at 37 C and pH 6 and the corresponding crude extracts were tested for their ability to neutralize the cytotoxic effect. Tests were carried out with nonadsorbed immune sera, as well as with immune sera adsorbed by TRU cells incubated at 37 C and pH 7. A typical neutralization test is shown in Table 1. The precipitate formed by mixing the immune sera with portions of crude pH 6 extract was discarded, and the supernatant fluids were tested for cytotoxic activity against rabbit and guinea pig monocytes. They were found either to be devoid of cytotoxic activity or to possess a markedly decreased activity; pH 6 antigen could not be detected by gel diffusion tests in these neutralized preparations. Normal rabbit and

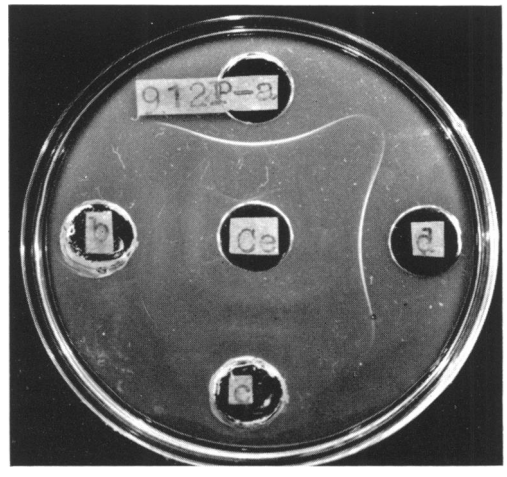


FIG. 1. (912) *Specificity of pH 6 antigen to TRU cells incubated at 37 C and pH 6 and to their crude extract. a: TRU (37 C, pH 6) living cells; b: TRU (37 C, pH 7) living cells; c: crude pH 7 extract; d: crude pH 6 extract; Ce (center): AI Se (rabbit immune serum prepared by injecting pH 6 crude extract and adsorbed with TRU (37 C, pH 7) living cells.*

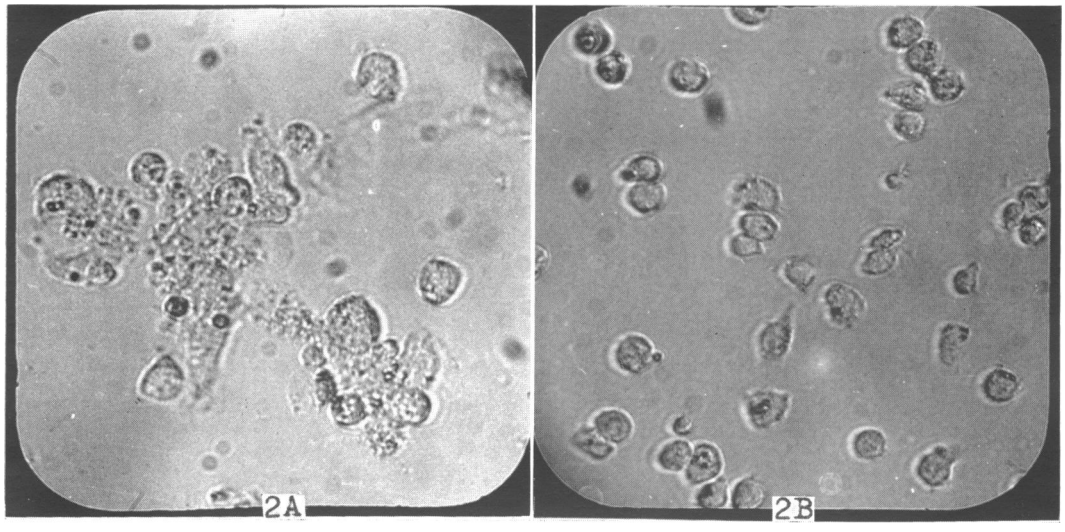


FIG. 2. *Cytotoxic effect of pH 6 antigen preparations on monocytes. A: degeneration of monocytes caused by the crude extract; B: normal layer of monocytes.*

guinea pig sera did not neutralize the cytotoxic activity.

*Agglutination of red blood cells.* The crude pH 6 extracts agglutinated red blood cells. This agglutination was irreversible, and the cells associated in one or more aggregates. Whole cells containing the pH 6 antigen caused the same effect. The unit of agglutinating activity was defined as the smallest quantity of protein ($\mu$g) that caused a clearly visible reaction. Specific activity was expressed in units of agglutinating activity per mg of protein. Activity of the pH 6 crude extracts ranged from 2 to 6 units per mg of protein. Whole cells of *P. pestis* incubated at 37 C and pH 7.0, 28 C and pH 7.0, and 28 C and pH 6, respectively, as well as crude extracts derived from them, did not agglutinate red blood cells in quantities up to 2.5 mg of protein. Neutralization experiments, carried out with the same sera and under the same conditions as used for neutralization of the cytotoxic effect, yielded equivocal results. In some experiments, a certain degree of neutralization was observed, whereas in other tests the immune sera either did not neutralize the agglutinating effect, or even produced an enhancement of the reaction.

Red blood cells of rabbits and guinea pigs were agglutinated under the same conditions as those described for sheep red blood cells.

It was found possible to adsorb the pH 6 antigen to red blood cells. For this purpose, blood cells were brought in contact with a sample of pH 6 crude extract. The aggregate formed upon treatment with the crude extract was separated from the remaining supernatant fluid, washed three times in isotonic buffered saline (pH 6.4), resuspended in a small amount of distilled water, and finally disrupted by maceration. It was found, by gel diffusion, that the pH 6 antigen was adsorbed to the aggregate, whereas the other antigens present in the crude preparation remained in the supernatant fluid. The aggregate, containing the pH 6 antigen, was cytotoxic for monocytes and induced the skin reactions to be described below. The supernatant fluid and the hemolyzed suspension of normal red blood cells were not cytotoxic for monocytes.

*Inflammatory reactions of the skin.* When the crude pH 6 extract was injected intradermally, swelling, edema, hemorrhages, and necrosis (diameter, 20 to 30 mm in marked positive cases) were produced (Fig. 3). These inflammatory reactions usually appeared 18 hr after injection, reached their maximum at 24 to 30 hr, and were still visible after 3 days. The specific activity of crude pH 6 extracts ranged from 1.6 to 3.3 units per mg of protein. This activity was specifically neutralized by immune serum. Good correlation was observed between the degree of cytotoxic activity shown by pH 6 crude extracts and their ability to induce skin reactions. The extracts prepared from *P. pestis* cells devoid of

TABLE 1. *Neutralization of cytotoxic effect by specific immune serum**

| Serial no. | Reactant mixture | Quantity of extract | Cytotoxic effect | |
|---|---|---|---|---|
| | | | Degree of reaction† | Units/mg of protein |
| | | μg | | |
| 1 | A I Se dilution | 1,000 | 3+ | 1.0 |
| 2 | (1:5) + pH 6 | 500 | 0 | |
| 3 | crude extract | 250 | 0 | |
| 4 | | 125 | 0 | |
| 5 | N R Se Dilution | 1,000 | 3+ | 8.0 |
| 6 | (1:5) + pH 6 | 500 | 3+ | |
| 7 | crude extract | 250 | 3+ | |
| 8 | | 125 | 2+ | |
| 9 | | 62 | 1+ | |
| 10 | pH 6 Crude ex- | 1,000 | 3+ | 8.0 |
| 11 | tract + phys- | 500 | 3+ | |
| 12 | iological saline | 250 | 3+ | |
| 13 | | 125 | 2+ | |
| 14 | | 62 | 1+ | |
| 15 | A I Se + saline | — | 0 | — |
| 16 | N R Se + saline | — | 0 | — |

* Reactants: crude pH 6 extract of strain TRU; A I Se: (see Fig. 1) I Se adsorbed by TRU cells incubated at 37 C, pH 7; N R Se: normal rabbit serum. The reactant mixtures were incubated for 3 hr at 37 C and kept at 4 C overnight. The precipitates formed in tubes 1, 2, 3, and to a lesser extent in tube 5 were discarded by centrifugation.

† Legend: 3+, complete destruction; 2+, partial destruction, formation of macroaggregates, some monocytes still normal; 1+, aggregates of monocytes, the majority of the cells in good condition; 0, no cytotoxic effect.

pH 6 antigen were not active when injected intradermally, even in large quantities.

*Fractionation and purification of the biologically active principles from the crude pH 6 extracts.* Attempts were made to purify the factor or factors responsible for the described biological activities. These attempts included ammonium sulfate precipitation, preparative electrophoresis, and calcium phosphate gel adsorption.

*Ammonium sulfate precipitation.* Fractions obtained by gradual precipitation with ammonium sulfate were examined for the presence of pH 6 antigen and for biological activity. Invariably, most of the biological activity and the pH 6 antigen were found in the fractions precipitated from 0 to 50% ammonium sulfate saturation. The recovery of protein within this range was 37 to 60%. Therefore, the fraction collected at 0 to 50% saturation was used for further studies. This fraction was called "ammonium sulfate precipitate." The course of precipitation is given in Table 2.

*Paper and immunoelectrophoresis.* The ammonium sulfate precipitate was examined by paper electrophoresis. Two to three fractions were separated by this procedure. Immunoelectrophoretic tests showed that one of the fractions, which migrated to the anode, was the pH 6 antigen. Therefore, it was thought that preparative electrophoresis of the ammonium sulfate precipitate might be useful as a further step in the purification.

*Preparative electrophoresis.* The results of a typical experiment are given in Fig. 4. Three peaks of protein were obtained, and one of them was identified as the pH 6 antigen. The eluate of this peak behaved electrophoretically as a homogenous fraction at pH values of 5.5 to 9.0, and exhibited all three biological activities described.

*Calcium phosphate gel adsorption.* The ammonium sulfate precipitate or the purified electrophoretic fraction were further fractionated by means of batch adsorption and elution; 60 to 80% of the protein fraction was adsorbed on the gel. Two procedures were used for eluting the protein adsorbed to the gel.

(i) The first procedure was used as a routine step in the purification. The course of the elution is given in Fig. 5. The elution was carried out by

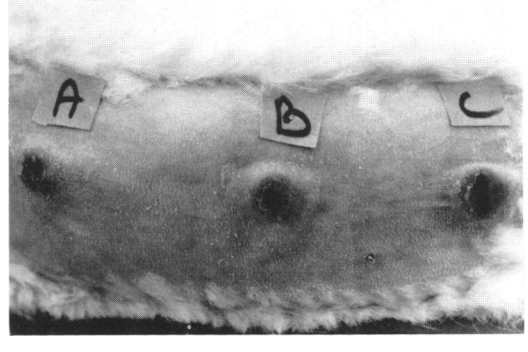


FIG. 3. *Skin inflammatory reaction caused by pH 6 antigen preparations. Quantities injected: A = 150 μg; B = 300 μg; C = 600 μg.*

TABLE 2. *Biological and immunochemical properties of $(NH_4)_2SO_4$ fractions prepared from crude pH 6 extract of strain TRU*

| Fraction (per cent saturation) | Total mg of protein | Per cent recovery | Biological activity (units/mg of protein) | | | Gel diffusion | |
|---|---|---|---|---|---|---|---|
| | | | Cytotoxic | Skin-reactive | Agglutinating red blood cells | pH 6 Antigen | Other lines |
| Crude | 140 | 100.0 | 5 | 4 | 2.3 | + | 3–4 |
| 0–20 | 60 | 42.8 | 18 | 9 | 3.7 | + | 2 |
| 20–30 | 15.5 | 11.1 | 14 | 11 | 3.6 | + | 2 |
| 30–50 | 9.0 | 6.4 | 4 | 5 | 2.0 | + | 2 |
| 50–70 | 9.9 | 7.1 | 1 | 0.7 | 0.5 | Nil or traces | 3 |
| Total recovery | 94.4 | 67.4 | | | | | |

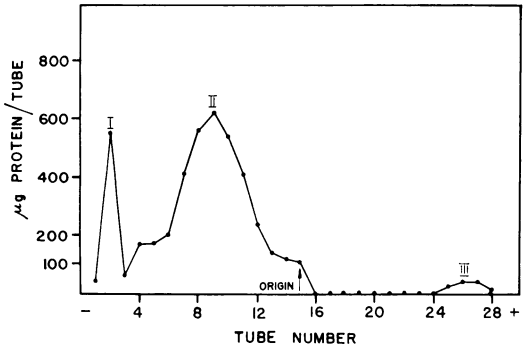


FIG. 4. *Electrophoresis of pH 6 antigen, ammonium sulfate precipitate, on Geon sodium barbital buffer (pH 8.6), 0.1 μ ionic strength. The pH 6 antigen, exhibiting all the biological activities described, was identified with peak II.*

increasing concentrations of 0.01, 0.05, and 0.2 M phosphate buffer (pH 6.8). Three distinct protein peaks could be separated. The first protein fraction eluted by 0.01 M phosphate buffer did not contain the pH 6 antigen or only traces of it and was devoid of biological activity. This fraction could only be detected when the ammonium sulfate precipitate was used as starting material. The second protein fraction was eluted by 0.05 M phosphate buffer and showed only the precipitation line of the pH 6 antigen in gel diffusion tests. This fraction had moderate cytotoxicity for monocytes, induced slight intradermal reaction, and did not agglutinate red blood cells. The 0.05 M phosphate buffer was replaced by 0.2 M phosphate buffer when the total quantity of protein in the eluate reached 500 μg. A third fraction was then eluted by 0.2 M phosphate buffer, which was also identified immunochemically as the pH 6 antigen. This fraction was highly cytotoxic for monocytes, caused marked skin inflammatory reaction, and agglutinated red blood cells. Thus, two protein fractions were obtained by this method, immunochemically identical but differing in spectrum and degree of biological activity. It was considered likely that more than one factor was responsible for the biological activities described, and this possibility was next investigated by

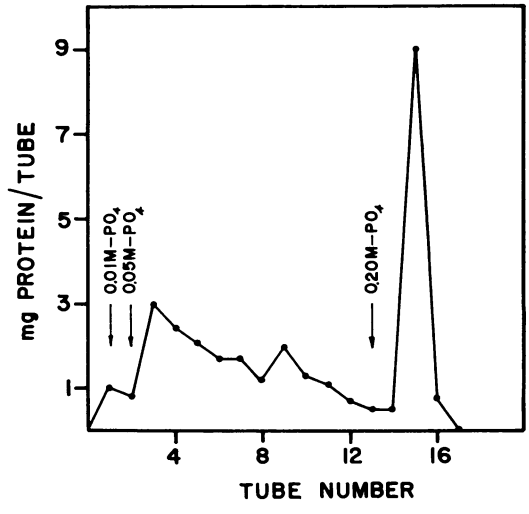


FIG. 5. *Elution of pH 6 antigen, ammonium sulfate precipitate, from calcium phosphate gel by phosphate buffer, pH 8 (procedure I): 0.05 M and 0.2 M eluates contained the pH 6 antigen, were cytotoxic for monocytes, caused skin reactions, and also agglutinated red blood cells.*

using a second procedure in which the course of elution was as shown in Fig. 6.

(ii) In the second procedure, the first elutions were also carried out with 0.01, 0.05, and 0.2 M phosphate buffer (pH 6.8). The only difference at this stage was that the elutions by 0.05 M phosphate buffer were continued until no more protein was detected in the eluates (less than 100 μg total quantity), and then the 0.2 M phosphate buffer was used for further elutions. No protein was eluted by the 0.2 M phosphate buffer under these conditions. Similarly, no protein was eluted further with buffers of higher ionic strength (0.5 and 1.0 M phosphate buffer at pH 6.8), or at higher pH and with lower ionic strength (0.01 M barbital buffer at pH 9.0 and 0.01 M sodium hydroxide glycine buffer at pH 10.1).

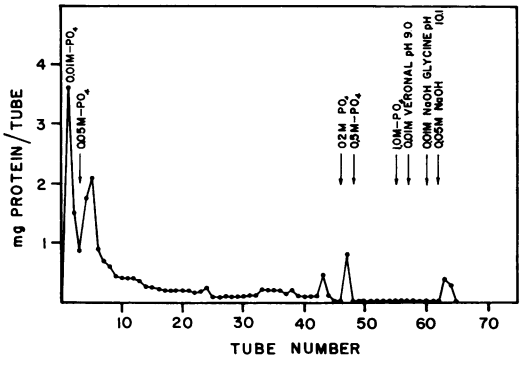


FIG. 6. *Elution of pH 6 antigen, ammonium sulfate precipitate, from calcium phosphate gel, by various buffers (procedure II). The pH 6 antigen was present in portions eluted by 0.05 M phosphate buffer and by 0.05 M sodium hydroxide. Only the portion eluted by 0.05 M sodium hydroxide agglutinated red blood cells.*

However, a protein fraction could be eluted by 0.05 M sodium hydroxide. This fraction was immediately neutralized and checked for biological activity. It agglutinated red blood cells, was cytotoxic for monocytes, and contained only the pH 6 antigen as shown by gel diffusion. The whole course of the purification is summarized in Table 3.

*Properties of the active fractions.* Some chemical and physical tests were performed to elucidate further the nature of the biologically active fractions.

*Phenol-water fractionation.* Since the Mollisch test had given a slightly positive reaction with crude extracts, the possible presence of a biologically active polysaccharide was further investigated. The material was treated by the method of Westphal, Luderitz, and Bister (1952), and it was found, after concentration, that the water phase, which contained trace amounts of polysaccharide, was biologically inactive.

*Acid precipitation and thermolability.* The pH 6 antigen could be precipitated from the crude extract at pH 2.6 to 5.5. The fraction precipitated and dissolved in 0.01 M phosphate buffer (pH 8.0) contained all the biological activities described. The crude extract lost all its biological activity by heating at 56 C for 30 min.

*Ultracentrifugal analyses.* The sedimentation behavior of two fractions, eluted from the calcium phosphate gel by the first procedure, was determined (Fig. 7). Both fractions were previously identified immunochemically as pH 6 antigen. One of the fractions was obtained by elution with 0.05 M phosphate buffer; it showed moderate cytotoxic and skin-reactive activity and did not agglutinate red blood cells. The sedimentation

TABLE 3. *Comparison of biological activity and gel diffusion pattern of pH 6 antigen preparations at different stages of fractionation*

| Nature of fraction | Gel diffusion | | Biological activity (units per mg of protein) | | | Per cent recovery |
|---|---|---|---|---|---|---|
| | pH 6 Antigen | Other lines | Cytotoxic | Skin-reactive | Agglutinating | |
| Crude extract | + | 3–4 | 5 | 2.3 | 4 | 100.0 |
| 50% ammonium precipitate | + | 2–3 | 12 | 3.1 | 8 | 60.3 |
| Geon electrophoresis | + | 0 | 27 | 9.0 | 29 | 24 |
| 0.05 M $CaHPO_4$ eluate (procedure I) | + | 0 | 6 | 2.0 | 0.7 | 7(17)* |
| 0.20 M $CaHPO_4$ eluate (procedure I) | + | 0 | 25 | 7.0 | 25 | 2(9)* |
| Crude pH 7 extract | 0 | 3–4 | 0.4 | 0.4 | 0.4 | |

* Per cent recovery when the starting material for $CaHPO_4$ gel adsorption was ammonium precipitate.

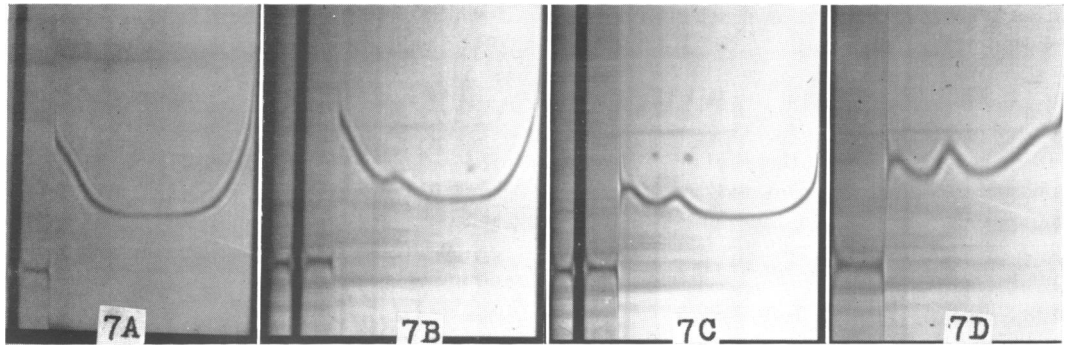


FIG. 7. *Sedimentation pattern of $CaHPO_4$ gel eluates; phosphate buffer (pH 6.8) at 56,100 rev/min. A: portion eluted by 0.05 M phosphate buffer (procedure I; sedimentation in 0.5 M phosphate buffer. Photograph taken 24 min after reaching maximal speed; optical angle, 50°. Only light component present. B, C, D: portion eluted by 0.2 M phosphate buffer (procedure I); photographs taken 20 min after reaching maximal speed; a heavy component (H) and a light component (L) were detected. B: sedimentation in 0.5 M phosphate buffer; optical angle 55°; areas ratio of $H(S_{20} - 10.4)/L(S_{20} - 1) = 0.3$. C: sedimentation in 0.2 M phosphate buffer; optical angle 50°; areas ratio of $H(S_{20} - 12.6)/L(S_{20} - 3.4) = 1.0$. D: sedimentation in 0.05 M phosphate buffer; optical angle 55°; areas ratio of $H(S_{20} - 14.3)/L(S_{20} - 2.9) = 1.6$.*

of this fraction was carried out in 0.5 M phosphate buffer, at a protein concentration of 3.3 mg per ml. There was no separation in the ultracentrifuge, thus excluding the presence of a heavy component. The second fraction tested was obtained by elution with 0.2 M phosphate buffer; it agglutinated red blood cells, was highly cytotoxic for monocytes, and induced marked skin reactions. The sedimentation of this fraction was carried out in 0.2 M phosphate buffer. At least two components were detected: a heavy component with $S_{20} = 12.6$ and a light component with $S_{20} = 3.4$. The ratio between the areas of the components was 1:1.

The same fraction was also sedimented at two additional phosphate buffer concentrations: 0.05 and 0.5 M. The ratio between the areas of the heavy and the light components was 1.6 at 0.05 M and 0.3 at 0.5 M phosphate buffer.

With the help of a fixed-partition cell, two fractions were isolated: one contained the light component only and the other contained a mixture of the heavy and the light components. The two fractions were tested immunochemically and biologically. Both fractions behaved in a similar manner electrophoretically (one band, with the same migration rate) and gave only the precipitation line of the pH 6 antigen. The separated light component possessed moderate cytotoxic activity (5 units per mg of protein) and did not agglutinate red blood cells in the maximal concentration tested (500 μg). The mixture of the heavy and light components had high cytotoxic activity (50 units per mg of protein) and agglutinated red blood cells (20 units per mg of protein).

## DISCUSSION

The course of infection with pathogenic microorganisms is characterized by their ability to cross the protective barrier of the host, resulting in bacteremia and the induction of damage to the tissues of the parasitized organism. In the case of *P. pestis*, the synthesis of at least three antigenic fractions has been related to the ability of the parasite to resist defense mechanisms of the host. One of these antigens is intimately associated with the surrounding envelope and has been described under various terms by Schutze (1932), Bhatnagar (1940), Amies (1951), Baker et al. (1947), Seal (1951), and Crumpton and Davies (1956). The other two antigens, named V and W, were described by Burrows and Bacon (1956) and Burrows (1957) and were associated by the same authors with the development of resistance to phagocytosis by polynuclear macrophages. A special form of plague bacilli, called M-type, which occurs in vivo and is highly resistant to phagocytosis by either neutrophils or monocytes, has been described by Burrows

(1955), Burrows and Bacon (1956), and Cavanaugh and Randall (1959).

The induction of damage to the tissues of the host by cells of *P. pestis* has been ascribed to the production of toxin (Schaer and Meyer, 1956; Packer, Rust, and Ajl, 1959; Cocking et al., 1960; Keppie, Smith, and Cocking, 1957). Another biological effect described by Chen and Meyer (1954) and Smith et al. (1960) is the inhibition of phagocytic activity in vivo by fraction I (capsular antigen) of *P. pestis*.

Various biological effects of *P. pestis* cells in vivo and in vitro, not related to specific antigens, also have been described by different authors. Thus, Domaradskü and Yarmyuk (1960) reported the lysis of human and animal blood fibrin; Eisler (1961) showed that some strains of *P. pestis* coagulated human plasma; Donovan et al. (1961) induced skin lesions in guinea pigs by intradermal inoculation of virulent *P. pestis* cells; Cavanaugh and Randall (1959) reported the intracellular multiplication of *P. pestis* in monocytes, followed by intense damage to the white cells.

Two of the biological effects attributed to whole cells of *P. pestis* (namely, induction of skin lesions and cytotoxicity for monocytes) have now, in our case, been produced by isolated extracts. In addition, we observed an agglutination of red blood cells as described in the present communication.

All three of these biological effects could be associated with the pH 6 antigen. Evidence for such association is as follows. Only extracts of *P. pestis* prepared from cells that contained the pH 6 antigen have shown the biological activities described in the present communication. In addition, immunochemically pure preparations of the pH 6 antigen have proven to be biologically active, and the association of the biological effects with pH 6 antigen was also demonstrated by the fact that two of the biological activities could be neutralized by immune sera prepared against the pH 6 antigen.

Differences have been observed between the hemagglutinating activity and the cytotoxic and inflammatory effects. Although the hemagglutinating activity was not neutralized, or only partially neutralized, by specific anti-pH 6 antigen immune serum, the other two biological properties were neutralized under such conditions. Three possibilities can be envisaged to explain the lack of neutralization of hemagglutinating activity: (i) two unrelated substances, one cytotoxic and inflammatory and related to the pH 6 antigen, and the other active on red blood cells, may be present in the crude pH 6 extracts; (ii) only one biologically active substance with two active sites may be involved; and (iii) a complex, defined immunochemically as the pH 6 antigen and containing at least two components, may be part of the crude pH 6 extract. At the moment, the best explanation for the results obtained is the probable presence of two components firmly bound in one complex in the crude pH 6 extract. One of the components (light fraction, cytotoxic and skin reactive) could be separated. However, attempts to isolate a fraction possessing only hemagglutinating activity and not containing the pH 6 antigen have been unsuccessful.

The sedimentation behavior of the complex at various buffer concentrations suggests that the molecules of light and heavy components may be combined in different ratios. The results obtained by the technique of calcium phosphate gel adsorption (by the second procedure used) showed that repeated elutions of the light component strengthened the link between the remaining complex and the gel.

The whole complex was more cytotoxic for monocytes and induced a more severe skin reaction than the isolated light component. It was also observed that aggregation of monocytes were more marked in the presence of the whole complex. It may be that the monocytes are more sensitive to destruction if first permitted to aggregate.

The thermolability of the biological activities described, and their absence in the water phase obtained by phenol-water extraction of crude preparations, suggests that the active biological principles are of a protein nature.

The biological activities which now have been observed to be associated with a surface antigen support the assumption, already formulated in a previous paper (Ben-Efraim et al., 1961), that synthesis of surface components at low pH may play an important role in the pathogenesis of plague.

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