

STUDIES ON IMMUNIZATION AGAINST PLAGUE

VI. GROWTH OF *PASTEURELLA PESTIS* AND THE PRODUCTION OF THE ENVELOPE AND OTHER SOLUBLE ANTIGENS IN A CASEIN HYDROLYZATE MINERAL GLUCOSE MEDIUM¹

ELLIS ENGLERBERG AND JUDITH BENTOFF LEVY

George Williams Hooper Foundation, University of California, San Francisco, California

Received for publication October 5, 1953

The immunizing capacity of various plague antigenic preparations for mice, monkeys, and man depends to a large extent on their Fraction I content (Baker *et al.*, 1952; Chen *et al.*, 1952). Fraction I is the designation given to that water-soluble antigenic portion of the plague bacillus that is soluble at 25 per cent saturated ammonium sulfate and precipitated at 33 per cent saturation. It appears to consist of two immunologically identical, but chemically different, fractions, IA and IB. Fraction IA is a protein-carbohydrate complex; Fraction IB is entirely protein (Baker *et al.*, 1952).

Since the present preparations for immunizing man against plague were found to be low in Fraction I and since present methods of preparing Fraction I are complicated and costly, involving growth of a virulent strain of *Pasteurella pestis* on a complex protein agar medium and subsequent extraction and fractionation (Baker *et al.*, 1947, 1952), simplified methods of growing *P. pestis* and of producing a preparation of high Fraction I content were sought.

Evidence also to be presented indicates that Fraction I is the envelope substance of *P. pestis* described by Rowland (1914) and Schütze (1932a). Since this is in disagreement with the findings of Amies (1951) to the effect that the one important antigen of *Pasteurella pestis* is a capsular substance which requires vigorous physical or chemical procedures to remove it from the cells, several pertinent experiments of his were repeated.

MATERIALS AND METHODS

Organism. *Pasteurella pestis*, strain A1122 (avirulent), originally isolated by Jawetz and

Meyer (1943), was employed for the most part in this study. This organism was selected because of its high soluble antigen² content when grown on a complex protein medium and its avirulence of long standing. An avirulent strain was chosen mainly on the basis of data that suggested that antigenically the major difference between virulent and avirulent strains of *P. pestis* is the quantity of Fraction I produced, not a so-called "virulence antigen" (Englesberg and Levy, 1953). Another consideration, of course, was the comparative simplicity of working with an avirulent strain rather than a virulent one and the possibility it presented for large-scale production.

Stock cultures of this strain were maintained initially on blood agar slants and then on casein hydrolyzate mineral glucose slants.

Medium. Since 37 C is the approximate optimum temperature for producing Fraction I (Chen *et al.*, 1952), a medium was sought that would support growth of *P. pestis* at this temperature. For growth in a mineral glucose medium at 30 C, *P. pestis*, strain A1122, requires phenylalanine, valine, isoleucine, methionine, and thio-sulfate (Englesberg, 1952). Such a medium failed to support growth of this organism at 37 C. According to Hills and Spurr (1952), *P. pestis* is much more demanding in its nutritional requirements when grown at 37 C than at 30 C, and different strains can be grown at 37 C in mixtures of from 8 to 15 different amino acids in some cases with (and in others without) the addition of biotin and pantothenate. They found hemin to be required for growth from small inocula at both 30 and 37 C, but there are now indications that this

¹ Sponsored by the Commission on Immunization of the Armed Forces Epidemiological Board and supported (in part) by the Office of the Surgeon General, Department of the Army, and the University of California.

² Soluble antigen refers to the antigen material of the plague bacillus extracted by saline at pH 7 or in the casein hydrolyzate mineral glucose medium during growth and forming a precipitate with an antiplague serum (Lederle serum).

requirement is an artifact of medium production (Englesberg, 1954).

Since there was the possibility that the entire culture could be used as the antigen complex or as a source of the antigen, it was desirable to employ a medium free of protein or of high molecular weight polypeptides. This would insure against the presence of any foreign sensitizing or toxic substances and would simplify any isolation procedure that might eventually be employed. On the basis of these considerations, attempts were made to grow *P. pestis* in a casein hydrolyzate mineral glucose medium (CHMG).

The composition of the initial or starting medium was casamino acid (Difco), 1 per cent (casein hydrolyzate); glucose, 0.2 per cent; NH_4Cl , 0.1 per cent; CaCl_2 , 10^{-3} per cent; FeCl_3 ($6\text{H}_2\text{O}$), 2.5×10^{-4} per cent; MgSO_4 ($7\text{H}_2\text{O}$), 0.05 per cent; and $\text{Na}_2\text{S}_2\text{O}_3$, 0.01 per cent. KH_2PO_4 and Na_2HPO_4 , pH 7, were added to yield a M/20 solution.

The casein hydrolyzate was autoclaved with distilled water, and the other components were added as sterile stock solutions. Sodium hydroxide was added subsequent to autoclaving to neutralize the casein hydrolyzate in all but a few preliminary experiments. Glucose was sterilized by filtering through sintered glass or autoclaving at 15 lb for 15 min as a 20 per cent solution. A concentrated solution of sodium thio-sulfate was sterilized by filtration, and the remaining inorganic compounds were autoclaved in concentrated solutions at 15 lb for 20 minutes.

Three different lots of casamino acids were used. With the lot used in the early experiments, obtained during World War II, a 3 per cent solution yielded optimum results. With more recently obtained lots of this material, use of a 4 per cent solution resulted in the production of a larger quantity of antigen than was obtainable with the older lot. Because of this difference, several experiments performed with the old lot in which the casein hydrolyzate content was crucial were repeated with the new lot. It would be repetitious to give the results obtained with both preparations, so in the case of these duplicate experiments only those results obtained with the presently obtainable casamino acid will be reported.

In the development of the optimum cultural conditions for growth and antigen production, the scale of the experimentation was important.

Since the purpose was to provide sufficient material for extensive animal and immuno-chemical investigation and at the same time to pave the way for large-scale pilot operation, it was decided to employ a method for exploring the optimum conditions for antigen production that could be used directly without further modification to provide large amounts of plague culture. This was provided for by employing a variable speed and eccentric rotory type shaking machine that would hold, for example, eighty 1 liter, wide mouth Erlenmeyer flasks. The speed and amplitude of rotation were varied and are indicated in each experiment. Duplicate 1 liter wide mouth Erlenmeyer flasks, each containing 250 ml of medium, were used, and results recorded are based on an average of determinations from both flasks.

pH. The pH of the cultures was determined at intervals by means of pH indicators.

Inoculum. The growth of an 18 to 24 hr casein hydrolyzate mineral glucose (3 per cent casein hydrolyzate) slant was washed off with 2.5 ml of sterile distilled water and subsequently diluted to 7.5 ml. Hormone agar slants were used in early experiments. One ml of this diluted suspension was used to inoculate each 250 ml of liquid medium. This provided an inoculum of about 5×10^6 viable cells per ml of medium.

Determination of the amount of growth. Total cell yields were measured at 24 hr intervals by the per cent light transmittance determined with a Coleman spectrophotometer at 650 μ . The blank employed consisted of the uninoculated liquid medium or a particular dilution thereof. A calibration curve for this instrument was established with a 3 day old slant culture of *P. pestis* suspended in saline. The total count of this suspension was determined with a Petroff-Hauser chamber. Microscopic observation of gram stained preparations was employed to detect contaminants.

Determination of the amount of soluble antigen produced. A modified semiquantitative turbidimetric precipitin test (Baker *et al.*, 1952) was performed on cell-free supernatants and in some cases on the saline washings of cells and saline extracts from acetone dried cells. Rabbit anti-Fraction I serum (Baker *et al.*, 1952) or Lederle Antiplague Immune Serum Globulin (Rabbit) prepared with living *P. pestis*, strain A1122,

was employed in this test to measure the soluble antigens.

In determining the quantity of antigen in the supernatants, 24 hr samples of the culture were centrifuged at 20,000 rpm for 20 min in a Spinco high speed centrifuge. The supernatant was collected and diluted serially. To one ml of two or more dilutions of the supernatant, one ml of a previously standardized serum was added. After the tubes had stood at room temperature for two hr or in the refrigerator overnight, one ml of saline was added, and the turbidity of the solution was read in a Klett-Summerson colorimeter using a blue filter. The amount of antigen per ml was calculated by means of a standard curve for the particular serum, which relates turbidity to quantity of antigen.

The one lot of antiplague serum (Lederle) employed was standardized arbitrarily with Fraction IA. One unit of soluble antigen is defined as the amount yielding the same turbidity with antiplague serum as one mg of Fraction IA.

Anti-Fraction I serum was standardized with Fraction IA prepared according to the method of Baker and his associates (1952).

In early experiments the procedure was to neutralize the supernatants prior to dilution and addition of the serum. This procedure was found to be unnecessary since turbidity was not affected by the pH of the medium.

Trichloroacetic acid (TCA) precipitable protein. To determine whether there was any correlation between total protein and soluble antigen in the supernatant (the latter determined with Lederle serum), to 5 or 10 ml of cell-free supernatant placed in a small centrifuge cup was added 1 or 2 ml of 60 per cent trichloroacetic acid, respectively. The suspension was stored overnight in the refrigerator, and the precipitate was spun down at 20,000 rpm for 20 minutes. The supernatant liquid was poured off, and the tube was drained dry. The precipitate was washed into a tared watch glass and dried to constant weight. Each determination was performed in duplicate. Blanks were prepared from casein hydrolyzate medium by the addition of trichloroacetic acid, following the procedure described above.

Toxicity. The varying of cultural conditions for optimum growth and antigen production afforded an excellent opportunity for studying the factors related to toxin production in *P. pestis*. Portions of the cell-free supernatant

medium, recovered at various intervals during the growth of *P. pestis* for the precipitin test, were used also to test for the presence of toxin. Five-tenths of a ml of various dilutions of the supernatant was inoculated intravenously into each of a group of Swiss mice. LD₅₀ was determined by the procedure of Reed and Muench (1938).

The "capsular" antigen of Amies. Amies' (1951) procedure for demonstrating the capsule of *P. pestis*, the dissolution of the capsule in the presence of potassium thiocyanate under controlled conditions, and the absence of the envelope in agar grown cells was duplicated. *P. pestis*, strain Tjiwidej, 1948, a strain similar to that employed by Amies, was used. The cells were scraped off the surface of agar plates and suspended in distilled water. The suspension was divided into two equal portions. To one were added potassium thiocyanate and potassium hydroxide to maintain a pH of 7.8, and to the other, only potassium hydroxide. Suspensions were incubated at 37 C under constant agitation. Precipitin tests described using Lederle antiplague serum and anti-Fraction I serum were performed immediately before the addition of potassium thiocyanate or potassium hydroxide and during the extraction procedure on cell-free supernatants prepared from samples of the cell suspension by centrifugation.

RESULTS

Growth and antigen distribution in 3 per cent casein hydrolyzate mineral glucose medium. The growth and amount of antigen soluble in the medium and remaining attached to the cells during 8 days of cultivation in a 3 per cent casein hydrolyzate (old lot) mineral glucose medium are shown in figure 1. The cultures were sampled at 24 hr intervals during incubation, and cell counts were determined directly on these samples or on 1:5 or 1:10 dilutions thereof, as was found necessary. Smears were made for microscopic examination, and pH of the cultures was determined. Ten ml of the samples were pipetted then into centrifuge cups and spun at 20,000 rpm for 20 minutes, and the supernatant fluids were assayed for antigen content. (The above procedure was followed routinely in most of the experiments to be described.) The recovered cells were resuspended in 5 ml of saline, placed in the refrigerator overnight, centrifuged, and the

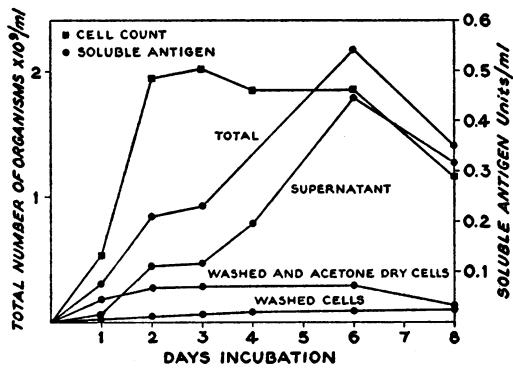


Figure 1. Growth of *Pasteurella pestis*, strain A1122, and distribution of soluble antigens in a 3 per cent casein hydrolyzate mineral glucose medium. Aeration: 95 rpm, 1 cm radius eccentric.

saline extract was collected and assayed for antigen content. The washed cells were rewashed then with small amounts of distilled water into glass centrifuge cups, dried by two successive washings with acetone at -60°C , and placed in a vacuum desiccator over sulfuric acid. The dried powder was suspended then with 5 ml of saline and the insoluble material spun down. The supernatant was collected and assayed for antigen content.

Maximum growth occurred on the third day of incubation and was followed by slow and then by rapid lysis. Antigen was released into the medium during this time, reaching a maximum on the sixth day of incubation. About 82 per cent of the antigen that could be removed from the cells was released into the supernatant by the sixth day; only an additional 4 per cent was removed by saline washings of the cells, and 14 per cent by a further acetone drying and saline extraction. A fairly constant amount of soluble antigen remained attached to the cells during the 8 day period, while the amount of antigen in the supernatant was large and increased progressively. Soluble antigen was produced in two distinct stages: the initial stage between 0 and 3 days of incubation, prior to cell lysis, and the secondary stage, which appears to depend on the lytic processes. In experiments in which both anti-Fraction I serum and Lederle anti-plague serum were used (figure 7) in assaying the antigen content of the supernatant fluid, it was demonstrated that these two stages of antigen production actually are related to the production of at least two different antigens. The first stage

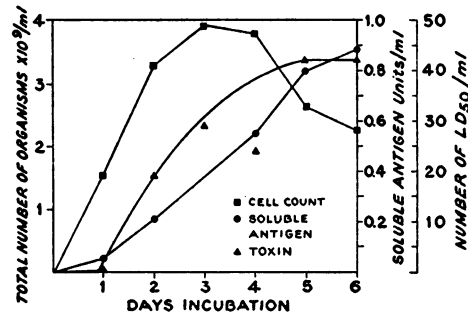


Figure 2. Growth of *Pasteurella pestis*, strain A1122, and soluble antigen and toxin production in a 3 per cent casein hydrolyzate mineral glucose medium. Aeration: 175 rpm, 1.5 cm radius eccentric.

is characterized by the production of Fraction I which reaches a peak at maximum growth and remains fairly constant during the cell lysis that follows. In the secondary or lytic stage, one or more antigens other than Fraction I, probably somatic in origin, are released.

The fact that the release of Fraction I into the medium is separated by a significant time interval from the release of other antigens indicated that it would be possible to determine the optimum conditions for the production of both these antigenic components of *P. pestis* simultaneously.

The ease with which plague antigens are released into the medium during the growth cycle indicated the plausibility of exploring the optimum conditions for soluble antigen production through assays on the supernatant fluid with antiplague serum. Although at 3 days of incubation (figure 1) only 50 per cent of the total soluble antigen (Fraction I) was found in the supernatant, while the remainder was attached to the cell, with the development of optimum cultural conditions the increased yield of Fraction I produced was found solely in the supernatant fluid (see Discussion). Since Fraction I and little else that yields a precipitate with this type of serum are released before cell lysis, Lederle serum can be employed at the point of maximum growth as a means of estimating Fraction I production and subsequently as a measure of total somatic antigen production.

Aeration. Employing the same medium as in the preceding experiment and increasing the aeration of the medium by raising the speed of

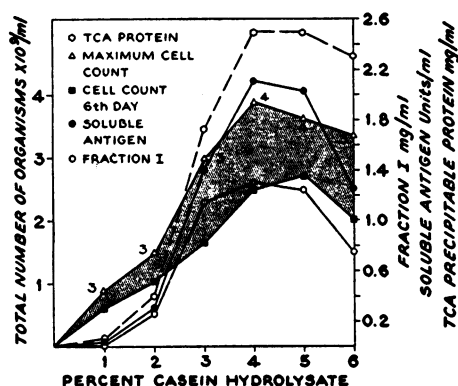


Figure 3. The relationship between the concentration of casein hydrolyzate and growth of *Pasteurella pestis*, strain A1122, soluble antigen and protein production. Medium: casein hydrolyzate concentration as indicated and the ingredients of the starting medium. Aeration: 175 rpm, 1.5 cm radius eccentric. Numbers on curve indicate the day of incubation at which maximum growth was reached. Shaded area indicates the amount of cell lysis.

rotation to 175 rpm and the eccentric to 1.5 cm in radius increased both the rate of growth and the antigen production as well as the total yields of antigen (figure 2). Cells lysed at the third day, and soluble antigen production reached a maximum at the sixth day. About 0.4 mg of Fraction I was in solution before lysis set in. Further increase in speed of rotation or radius of the eccentric or reducing volume of medium from 250 to 100 ml did not further increase growth or antigen production.

Toxin production. The toxicity of the cell-free supernatant medium was tested during the variation of concentrations of casein hydrolyzate (old lot), glucose, phosphate buffer, pH, and iron. In all cases the same general pattern was observed. A typical example of the relation of growth and toxin and antigen production is shown in figure 2. The toxicity of the supernatant medium increased during the active growth, the stationary phase, and during cell lysis. Total toxin production was always directly correlated with the amount of cell growth. The supernatant is detoxified by adding 0.1 to 0.2 per cent formalin. Further substantiation that the toxicity of the supernatant is not the result of simple intermediary metabolic products of *P. pestis* was shown by the toxicity of dialyzed supernatant and by the recovery of the toxin by $(\text{NH}_4)_2\text{SO}_4$

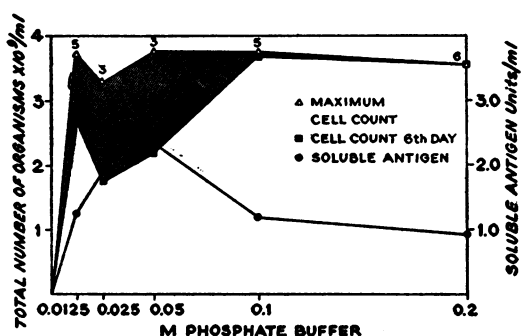


Figure 4. Relationship between concentration of phosphate buffer and growth of *Pasteurella pestis*, strain A1122, and soluble antigen production. Medium: 4 per cent casein hydrolyzate and the ingredients of the starting medium. Aeration: 175 rpm, 1.5 cm radius eccentric. Numbers on curve indicate the day of incubation at which maximum growth was reached. Shaded area indicates the amount of cell lysis.

precipitation and subsequent dialysis (Englesberg and Levy, 1953).

Variation in the concentration of casein hydrolyzate³ (figure 3). Growth, soluble antigen, and Fraction I production were maximal with 4 per cent casein hydrolyzate. Maximum growth (3.92×10^9 cells per ml) occurred on the fourth day of incubation and was followed by lysis of approximately 1.4×10^9 cells per ml. The maximum amount of soluble antigen (2.1 units per ml of supernatant) was produced on the sixth day of incubation, while the maximum amount of Fraction I (1.3 mg per ml) was found at 3 days' incubation and declined slightly thereafter. Total plague protein in solution is correlated directly with the amount of total soluble antigen.

Variation in the concentration of phosphate buffer (figure 4). Although changing the concentration of phosphate buffer from 0.0125 M to 0.2 M did not significantly affect the total cell yield, growth lagged appreciably with concentrations over 0.05 M and below 0.025 M. At concentrations below 0.025 M, acid production, indicated by the low pH, was probably the cause of the delay in growth. With the higher concentrations (0.1 and 0.2 M), cells did not lyse by the sixth day; lysis was massive in flasks containing 0.05 M phosphate. The fact that pH varied

³The casamino acid (Difco) (casein hydrolyzate) used in this and in the following experiments is material currently available.

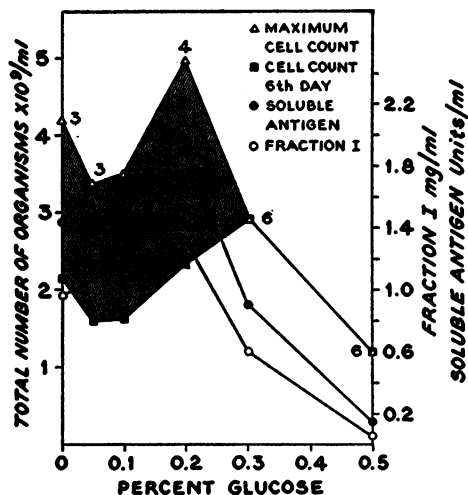


Figure 5. Relationship between concentration of glucose and growth of *Pasteurella pestis*, strain A1122, and antigen production. Medium: 4 per cent casein hydrolyzate and the ingredients of the starting medium. Numbers on curve indicate the day of incubation at which maximum growth was reached. Shaded area indicates the amount of cell lysis.

insignificantly during growth with these concentrations eliminates pH as the factor responsible for lysis. The optimum phosphate concentration for Fraction I and soluble antigen production is 0.05 M (as in the starting medium).

Variation in the concentration of glucose (figure 5). Growth and antigen production were maximal when the 4 per cent casein hydrolyzate mineral glucose starting medium contained 0.2 per cent glucose. Higher concentrations inhibited growth due to excessive acid production. Although media without glucose showed little growth of *P. pestis* during the first 24 hr (not shown on the graph), in comparison to those containing from 0.05 to 0.2 per cent glucose, the total cell crop was higher in the medium containing no glucose than in that containing 0.05 or 0.1 per cent. This may be the result of selection of a mutant capable of utilizing the casein hydrolyzate as primary carbon source in the medium lacking glucose and inhibition of such selection due to growth of the wild type in the presence of a low concentration of glucose (Ry 24 and Schneider, 1948).

Variation in pH. Duplicate flasks of casein hydrolyzate were autoclaved in distilled water and subsequently brought up to pH 6.5, 6.8, 7.0, 7.2, and 7.8 with sterile sodium hydroxide.

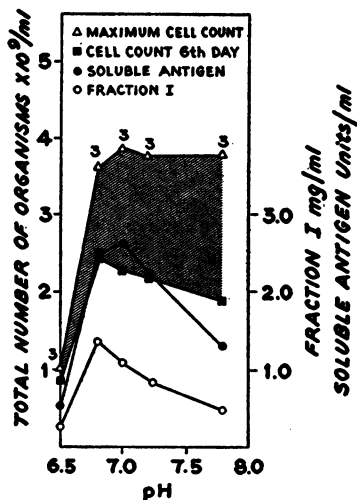


Figure 6. Relationship between pH and growth of *Pasteurella pestis*, strain A1122, and antigen production. Medium: 4 per cent casein hydrolyzate and the ingredients of the starting medium. Aeration: 175 rpm, 1.5 cm radius eccentric. Shaded area indicates the amount of cell lysis.

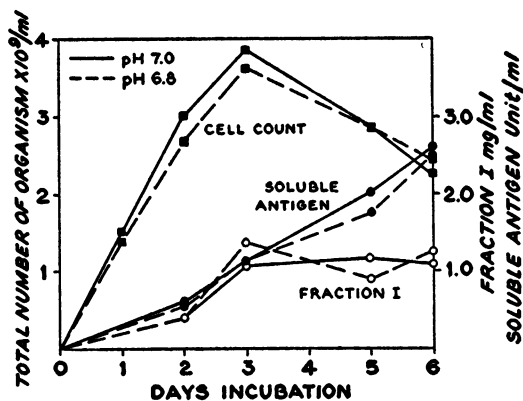


Figure 7. Growth of *Pasteurella pestis*, strain A1122, and antigen production in 4 per cent casein hydrolyzate medium and the ingredients of the starting medium. Initial pHs 6.8 and 7.0. Aeration: 175 rpm, 1.5 cm radius eccentric.

Phosphate buffer at these pHs and the other ingredients of the starting medium were added to the respective flasks. The yield of cells and of total soluble antigen was maximal at pH 7 (figures 6 and 7). Although the amount of Fraction I was slightly larger when the initial pH of the medium was buffered at pH 6.8 than at 7.0, the difference does not appear to be significant (figure 7). This was verified further by several repetitions of this experiment with me-

dium buffered at these two levels. Although with an initial pH of 7.8 growth was only slightly less than at pH 7 and the amount of lysis was greater, much less Fraction I and total soluble antigen were produced. Whether this is the result of denaturation of the plague antigen due to excessive alkalinity or to the failure of the organism to produce these antigens under these circumstances has not been determined. Therefore it appears that besides cell yield and temperature of incubation (Chen *et al.*, 1952), the initial pH of the medium is an important factor governing antigen production in *P. pestis*.

Variation in minerals and simplification of the medium. So far it has been demonstrated that a 4 per cent casein hydrolyzate, 0.2 per cent glucose medium, buffered at pH 7.0 with *m*/20 phosphate buffer yields maximal growth and soluble antigen production. The 4 per cent casein hydrolyzate solution contains, per ml, approximately 0.053 micromoles of magnesium, 0.025 micromoles of calcium, and 0.004 micromoles of iron. (This information was obtained from Dr. H. W. Schoenlein of Difco Laboratories.) In preparing the starting medium, the mineral content was increased by adding two micromoles of magnesium, 0.092 micromoles of calcium, and 0.0092 micromoles of iron per ml of medium. In addition, 18.8 micromoles of NH_4^+ and 0.8 micromoles of sulfur (as $\text{S}_2\text{O}_7^{2-}$) per ml were added, the former to spare the casein hydrolyzate and the latter as a source of cystine (Englesberg, 1952).

To determine whether the addition of these minerals was essential, each was omitted in turn. The results indicated that the addition of magnesium is essential for growth of *P. pestis* and for antigen production. The omission of ammonium chloride appeared to enhance growth but not because of its effect on the pH. The addition of iron, calcium, or $\text{S}_2\text{O}_7^{2-}$ salts appeared to have little effect.

Since magnesium might be limiting growth and antigen production, the optimum amount to be added to the medium was ascertained by varying its concentration from 0 to 8 micromoles per ml. Figure 8 indicates that the amount of magnesium added in the starting medium was indeed insufficient and that the optimum concentration of magnesium is at least twice that amount (4 micromoles per ml).

On the basis of this finding it was then necessary to determine whether, with the increase in

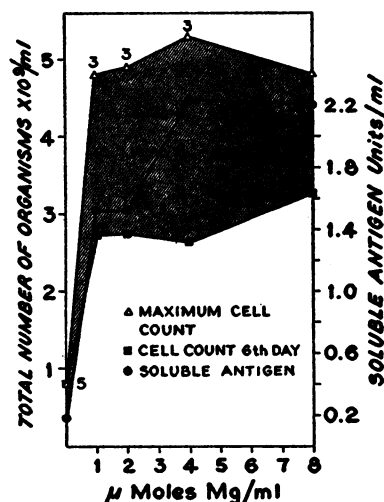


Figure 8. Relationship between magnesium concentration and growth of *Pasteurella pestis*, strain A1122, and antigen production. Medium: 4 per cent casein hydrolyzate and the ingredients of the starting medium. Aeration: 175 rpm, 1.5 cm radius eccentric. Numbers on curve indicate the day of incubation at which maximum growth was reached. Shaded area indicates the amount of cell lysis.

the concentration of magnesium, some other mineral, glucose, or casein hydrolyzate might be limiting growth. The concentration of glucose, casein hydrolyzate, iron, calcium, and ammonium chloride was varied separately in the presence of 4 micromoles of magnesium (other ingredients as in the starting medium). These experiments demonstrated that 0.2 per cent glucose and 4 per cent casein hydrolyzate remained optimum for growth and antigen production. No requirement for additional iron or calcium or NH_4^+ over that in the casein hydrolyzate was demonstrated.

In the development of this medium the casein hydrolyzate was autoclaved separately, and the magnesium sulfate and other salts, glucose, phosphate buffer, and sodium hydroxide were subsequently added. Further experiments have demonstrated that the casein hydrolyzate could be autoclaved with the mineral salts without any ill effect. Autoclaving the casein hydrolyzate without phosphate and sodium hydroxide is beneficial for initiating growth from small inocula. However, using an inoculum of 5×10^6 viable cells per ml of medium gave optimum yields

TABLE 1
Comparison of the Fraction I and total soluble antigen content of plague vaccines

VACCINES*	TOTAL SOLUBLE ANTIGEN	FRACTION I
	units/ml	mg/ml
Cutter Laboratory†	0.44	0.28
Haffkine Institute†	0.57	0.68
CHMG supernatant 1	0.52	
CHMG supernatant 2	1.08	
CHMG supernatant 3	1.14	1.36
CHMG supernatant 4	2.50	1.24

* Cutter Laboratory Plague Vaccine is a formalin killed suspension of virulent plague bacilli grown at 37 C on hormone agar.

Haffkine Institute Plague Vaccine is a formalin killed casein hydrolyzate culture of virulent plague bacilli probably grown at 28 C.

Casein hydrolyzate mineral glucose (CHMG) supernatants 1 and 2 are from 6 day old CHMG cultures of *Pasteurella pestis*, strain A1122 (avirulent), grown under suboptimum conditions.

Casein hydrolyzate mineral glucose supernatant 3 is from a 3 day old CHMG (optimum medium and conditions) culture of *Pasteurella pestis*, strain A1122 (avirulent).

Casein hydrolyzate mineral glucose supernatant 4 is a supernatant of a 6 day old culture from which supernatant 3 was obtained.

† Cells were spun down and precipitin tests were performed on the supernatant fluid.

when all ingredients (except glucose) were autoclaved together with the casein hydrolyzate.

Comparison of the antigen content of various plague vaccines with that of the casein hydrolyzate mineral glucose supernatants. Four different casein hydrolyzate mineral glucose supernatants were compared with the Cutter Laboratories Plague Vaccine and with the Haffkine Institute Plague Vaccine (table 1). Supernatants 1 and 2 were harvested from cultures of *P. pestis*, strain A1122, grown in suboptimum media. Supernatants 3 and 4 were taken from the same culture grown under optimum conditions. Supernatant 3 was harvested after incubation for 72 hr; supernatant 4 was harvested after 144 hr. Casein hydrolyzate mineral glucose supernatants 3 and 4 contained about twice as much Fraction I as the Haffkine vaccine and about 5 times as much as the Cutter vaccine. Supernatant 4 had about twice the amount of total soluble antigen that supernatant 3 had. This represents an in-

crease in somatic antigen content as a result of cell lysis.

DISCUSSION

The medium finally arrived at for optimum growth and soluble antigen production is simpler and showed more than a 10-fold increase in Fraction I and somatic antigen production and a 4- to 5-fold increase in total cell yield, as compared with the starting medium (table 2).

Growth of *P. pestis*, strain A1122, reaches a maximum (4 to 5 × 10⁸ organisms per ml), under the optimum conditions developed, on the third day of incubation, and this is followed by massive cell lysis which ceases by the sixth day. At least three different components of the plague bacillus are released into the medium during this growth cycle: Fraction I, somatic antigen(s), and toxin. Fraction I is released continuously during the logarithmic phase of growth, and a maximum concentration of about 1.0 to 1.4 mg per ml of supernatant fluid is produced coincident with the maximum yield of cells. Little, if any, additional Fraction I is released during the lytic period. To produce Fraction I in solution relatively free of somatic antigens, a 3 day old culture of *P. pestis*, grown under the conditions described, appears optimum. The somatic antigens are released during the cell lysis which occurs from the fourth to the sixth day. Yields of somatic antigen can be determined by differences in amounts of total soluble antigen and Fraction I as estimated by turbidimetric precipitin tests, using Lederle and anti-Fraction I serums, respectively, and are only approximations. Although Lederle serum may form a precipitate with plague toxin, the amount of toxin-antitoxin precipitate formed is insignificant (Englesberg and Levy, 1953). Thus, the precipitin test described measures antigenic material other than plague toxin in the supernatant. Small amounts of toxin, determined by *in vivo* test, are released during both cell growth and lysis, indicating that the plague toxin cannot be classified satisfactorily as an endotoxin in the old sense.

Growth and antigen production have varied somewhat in experiments undertaken at different times under presumably the same conditions. For this reason, in developing the optimum medium, the best medium arrived at in one series of experiments was always employed during the

TABLE 2
Comparison of the starting and optimum casein hydrolyzate mineral glucose media

INGREDIENTS	STARTING MEDIUM	OPTIMUM MEDIUM
Casein hydrolyzate (casamino acid (Difco))....	1.0%	4%
Glucose.....	0.2%	0.2%
MgSO ₄ ·7H ₂ O.....	0.05%	0.1%
FeCl ₃ ·6H ₂ O.....	2.5 × 10 ⁻⁴ %	
CaCl ₂	1.0 × 10 ⁻³ %	
NH ₄ Cl.....	0.1%	
Na ₂ S ₂ O ₃	0.01%	
KH ₂ PO ₄ —Na ₂ HPO ₄	pH 7, M/20	pH 7, M/20
Aeration.....	95 rpm	175 rpm
	1 cm radius (eccentric)	1.5 cm radius (eccentric)
YIELD		
Total cell count.....	1 × 10 ⁸ /ml	4–5 × 10 ⁸ /ml
Fraction I.....	Less than 0.1 mg/ml	1.0–1.4 mg/ml
Other antigens (somatic).....	Less than 0.1 unit/ml	Approx 1 unit/ml

Sodium hydroxide is added to neutralize the casein hydrolyzate prior to the addition of the phosphate buffer.

next series so as to establish a reliable base line with which to judge the results. This variation may be attributable to fluctuations in temperature of incubation (37 ± 1 C) (Hills and Spurr, 1952).

Early experiments (figure 1) indicated that on the third day of incubation about 50 per cent of the total soluble antigens extractable from *P. pestis* had been released into the supernatant fluid; the remainder was removed from the cells by saline extraction and subsequent acetone drying and extraction. A comparison of the distribution of Fraction I in a culture grown under the optimum conditions described, however, reveals that 86 per cent of the Fraction I produced is in the supernatant fluid, an additional one per cent is removed by a saline extraction of the cells, and the remainder is removed by saline extraction of acetone dried cells. The amount of antigen remaining attached to the cells appears to be fairly constant. Thus, the increase in yield of Fraction I by *P. pestis*, strain A1122, by growth under optimum conditions is indicated only in the supernatant fluid.

Observations on the ease with which Fraction I is released into the medium during the active growth of *P. pestis* appear similar to those of Dochez and Avery (1917) with regard to the release into the culture medium of the soluble antigen of the pneumococcus and give further

support to the identity of Fraction I as a mucoid substance surrounding the plague bacillus. This mucoid substance, termed an envelope, is distinguishable from a capsule in that it appears in the form of an exudate embedding a row of organisms in a continuous mass of slime. It is irregular in outline and density and is not sharply demarcated from the surrounding medium (Rowland, 1914; Schütze, 1932a; Bhatnagar, 1940).

Evidence from a number of sources indicates that Fraction I is this envelope substance of *P. pestis*, or at least that it comprises a good portion of it. Both Fraction I and the envelope are produced in large amounts at 37 C and in smaller amounts at lower temperatures (Schütze, 1932b; Bhatnagar, 1940; Chen *et al.*, 1952). All virulent and protective avirulent strains of *P. pestis* possess large envelopes and produce large quantities of Fraction I, while all nonprotective avirulent strains lack the envelope or possess a very diminished one and produce little Fraction I (Schütze, 1932a; Bhatnagar, 1940; Meyer, 1950; Englesberg and Levy, 1953). Protection of laboratory animals against plague is related to the amount of anti-envelope and anti-Fraction I present in the serum (Schütze, 1932b; Chen *et al.*, 1952). Antiserum prepared with rabbits or monkeys with Fraction I specifically alters the envelope of virulent plague bacilli. An immune

serum deprived of Fraction I antibody by specific adsorption fails to form aggregates on the envelope (Meyer, 1950).

Sokhey (1940) and recently Amies (1951) have attempted to demonstrate the existence of a true capsule and the lack of any mucoid envelope in *P. pestis*. Observations indicated that vigorous physical or chemical procedures were necessary to loosen the capsule and that the capsular material was highly antigenic (Amies, 1951). These findings of Amies appear to be in conflict with the evidence cited above as to the presence of Fraction I as the easily soluble envelope substance surrounding *P. pestis*.

To test the possibility that because of its great solubility the envelope substance (Fraction I) had been overlooked by Amies (1951), an experiment of his (designed to demonstrate the presence of the capsule, its dissolution with potassium thiocyanate, and the absence of an envelope, using agar grown cells) was repeated. This experiment demonstrated that 96 per cent of the Fraction I and 89 per cent of the total extractable soluble antigens were in solution in the distilled water immediately before the "extraction" process was begun. Small additional amounts of Fraction I and other antigens were liberated during the "extraction" procedure. Four per cent additional Fraction I was liberated both with cells incubated in H₂O at pH 7.8 and with those incubated in potassium thiocyanate. Eleven per cent additional total soluble antigen was liberated from the cells suspended in H₂O (pH 7.8), while only an additional 8 per cent of soluble antigen was released from those cells suspended in potassium thiocyanate. Results were similar with cell suspensions in saline at pH 7.8.

India ink preparations of the suspensions before the extraction procedure did show a definite halo around the bacilli, and this diminished on incubation. However, a similar preparation, made by emulsifying a portion of the stringy growth of *P. pestis* directly from the agar surface with a small amount of India ink, revealed no distinct capsules but masses of slime in different irregular patterns encompassing many bacilli.

Evidence, therefore, points to the existence of a highly soluble gelatinous envelope surrounding the cells of *P. pestis*. This has been confirmed recently by electron microscopy (Crocker *et al.*, 1954). The envelope dissolves very readily,

leaving behind a small amount of a material which microscopically appears similar to the typical bacterial capsule. The latter substance goes into solution with difficulty, perhaps as a result of its close proximity to the bacterial cell wall.

The question arises as to whether there is any distinction between the easily soluble gelatinous material (Fraction I or the envelope substance) and the "capsular" substance remaining attached to the cell. It seems at first glance that the comparison of the Amies antigen and Fraction I might lead to an answer to this question. Webster (1953) and Landy (1953) have just recently found purified Amies antigen to be identical to Fraction IA by various physical, chemical, and immunological tests, thereby substantiating our conclusions. Judging from the work of Baker and his colleagues (1947, 1952), Fraction IB is probably present also in the crude Amies preparation. However, as has been demonstrated, since Fraction I goes into solution so readily, it no doubt would contaminate the smaller quantities of the more difficult to extract "capsular" material. In fact, it is likely that the antigen isolated by the potassium thiocyanate extraction method may not be related at all to the capsule which is subsequently dissolved. The increased yield of Fraction I with extraction seems to indicate that the "capsular" material of Amies may indeed be additional Fraction I held somewhat tenaciously to the cell wall. However, since antigens other than Fraction I are extracted at the same time, one cannot be sure.

In conclusion, therefore, since the high protein content of Fraction I makes it unlikely that it could have originated as a modification of the cell wall (the criterion set down for a true capsule by Etinger-Tulczynska, 1933) and on morphological grounds as well, being irregular in shape and density and having no real boundary (Klieneberger-Nobel, 1948; Knaysi, 1951), Fraction I must be considered as the gelatinous envelope or slime layer of *P. pestis*. On a morphological basis *P. pestis* appears to possess a capsule as well. The question of whether or not the plague bacillus has a capsule in the sense defined by Etinger-Tulczynska (1933), and therefore differing chemically from the envelope substance, will have to await further investigation.

The Haffkine Institute, Bombay, India, has been producing a plague vaccine using a formalin

killed casein hydrolyzate culture of a virulent strain of *P. pestis* (Sokhey *et al.*, 1950). An analysis of one sample of this vaccine indicates that it contained 0.64 mg of Fraction I per ml of vaccine supernatant, about half as much as contained in the casein hydrolyzate mineral glucose supernatant described in this report. Mouse immunization experiments to be reported in a subsequent paper (Englesberg *et al.*, 1954) demonstrated that the greater amount of Fraction I in the casein hydrolyzate mineral glucose supernatant over that contained in the Haffkine vaccine is reflected by greater protection of mice against plague by the casein hydrolyzate mineral glucose supernatant. Seal and Mukherji (1950) have suggested several modifications of the medium employed in producing the Haffkine vaccine. These include the addition of phosphate buffer, calcium, magnesium, iron, and liver extract to the medium. Whether these modifications were employed in producing the vaccine analyzed in this study could not be determined. However, an indication of the comparative antigen yield in the modified medium suggested by Seal and Mukherji (1950) and that achieved in the casein hydrolyzate mineral glucose medium might be ascertained by comparing the cell yields in both media. Seal and Mukherji (1950), culturing at 28 C (temperature optimum for growth and suboptimum for Fraction I production), reported a maximum of 4.5×10^8 viable cells per ml of medium at 48 hr incubation. Using the medium described in this paper at 37 C (optimum for Fraction I production and suboptimum for growth), more than 3 times that number of viable cells was produced. The relatively poor growth reported by these investigators may be the result of insufficient aeration and a limiting carbon source.

ACKNOWLEDGMENTS

We would like to express our thanks to Miss L. Foster and Mrs. F. Scott for the mouse toxicity determinations, to Dr. T. H. Chen for preparations of Fraction IA, and to Mr. R. Muir for supplying anti-Fraction I serum.

SUMMARY

A casein hydrolyzate mineral glucose medium and cultural conditions have been developed for optimum growth of *Pasteurella pestis*, strain A1122 (avirulent), for the production of Fraction

I and somatic antigen. Maximum growth of *P. pestis* (4 to 5×10^9 organisms per ml) occurs on the third day of incubation and is followed by massive cell lysis which ceases by the sixth day. At least three different components of the plague bacillus are released into the medium during the growth cycle: Fraction I, somatic antigen(s), and toxin. Fraction I is released continuously during the logarithmic phase of growth, and a maximum concentration of about 1.0 to 1.4 mg per ml of supernatant fluid is produced by the third day. The somatic antigens are released during the lytic phase, reaching a maximum at the conclusion of cell lysis. Toxin is released both during cell growth and during lysis. The antigen content of the casein hydrolyzate mineral glucose supernatant is shown to be higher than that in other vaccines tested. Evidence points to the existence of Fraction I as the highly soluble gelatinous envelope surrounding the cells of *P. pestis*. The relationship between this substance, the Amies antigen, and the capsule of *P. pestis* is discussed.

REFERENCES

- AMIES, C. R. 1951 The envelope substance of *Pasteurella pestis*. *Brit. J. Exptl. Pathol.*, **32**, 259-273.
- BAKER, E. E., SOMMER, H., FOSTER, L. E., MEYER, E., AND MEYER, K. F. 1947 Antigenic structure of *Pasteurella pestis* and the isolation of a crystalline antigen. *Proc. Soc. Exptl. Biol. Med.*, **64**, 139-141.
- BAKER, E. E., SOMMER, H., FOSTER, L. E., MEYER, E., AND MEYER, K. F. 1952 Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. *J. Immunol.*, **68**, 131-145.
- BHATNAGAR, S. S. 1940 Bacteriological studies on *Pasteurella pestis* and *Pasteurella pseudotuberculosis*. I. The morphology, the growth and the dissociation of *Pasteurella pestis*. *Indian J. Med. Research*, **28**, 1-15.
- CHEN, T. H., QUAN, S. F., AND MEYER, K. F. 1952 Studies on immunization against plague. II. The complement-fixation test. *J. Immunol.*, **68**, 147-158.
- CROCKER, T. T., CHEN, T. H., AND MEYER, K. F. 1954 *Unpublished data*.
- DOCHEZ, A. R., AND AVERY, O. T. 1917 The elaboration of specific soluble substance by pneumococcus during growth. *J. Exptl. Med.*, **26**, 477-493.

- ENGLESBERG, E. 1952 Irreversibility of methionine synthesis from cysteine in *Pasteurella pestis*. *J. Bact.*, **63**, 675-680.
- ENGLESBERG, E. 1954 *Unpublished data*.
- ENGLESBERG, E., AND LEVY, J. B. 1953 *Unpublished data*.
- ENGLESBERG, E., LEVY, J. B., FOSTER, L. E., AND MEYER, K. F. 1954 *Unpublished data*.
- ETINGER-TULCZYNSKA, R. 1933 Bakterienkapseln und Quellungsreaktion. *Z. Hyg. Infektionskrankh.*, **114**, 769-789.
- HILLS, G. M., AND SPURR, E. D. 1952 The effect of temperature on the nutritional requirements of *Pasteurella pestis*. *J. Gen. Microbiol.*, **6**, 64-73.
- JAWETZ, E., AND MEYER, K. F. 1943 Avirulent strains of *Pasteurella pestis*. *J. Infectious Diseases*, **73**, 124-143.
- KLIENEBERGER-NOBEL, E. 1948 Capsules and mucoid envelopes of bacteria. *J. Hyg.*, **46**, 345-348.
- KNAYSI, G. 1951 *Elements of bacterial cytology*. 2nd Edition. Comstock Publishing Co., Ithaca, N. Y.
- LANDY, M. 1953 Reported at the 7th annual meeting of the Commission of Immunization, Armed Forces Epidemiological Board.
- MEYER, K. F. 1950 Immunity in plague: A critical consideration of some recent studies. *J. Immunol.*, **64**, 139-163.
- REED, L. J., AND MUENCH, H. 1938 A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**, 493-497.
- ROWLAND, S. 1914 The morphology of the plague bacillus. *J. Hyg., Plague Suppl.* III to vol. **13**, 418-422.
- RYAN, F. J., AND SCHNEIDER, L. K. 1948 The consequences of mutation during the growth of biochemical mutants of *Escherichia coli*. I. The pattern of adaptation. *J. Bact.*, **56**, 699-708.
- SCHÜTZE, H. 1932a Studies in *B. pestis* antigens: I. The antigens and immunity reactions of *B. pestis*. *Brit. J. Exptl. Pathol.*, **13**, 284-288.
- SCHÜTZE, H. 1932b Studies in *B. pestis* antigens: III. The prophylactic value of the envelope and somatic antigens of *B. pestis*. *Brit. J. Exptl. Pathol.*, **13**, 293-298.
- SEAL, S. C., AND MUKHERJI, S. P. 1950 Hydrolysate of casein as a fluid medium for the growth of *Pasteurella pestis*. *Ann. Biochem. and Exptl. Med. (India)*, **10**, 80-97.
- SOKHEY, S. S. 1940 The capsule of the plague bacillus. *J. Pathol. Bacteriol.*, **51**, 97-103.
- SOKHEY, S. S., HABBU, M. K., AND BHARUCHA, K. H. 1950 Hydrolysate of casein for the preparation of plague and cholera vaccines. *Bull. World Health Organ.*, **3**, 25-31.
- WEBSTER, M. E. 1953 Reported at the 7th annual meeting of the Commission on Immunization, Armed Forces Epidemiological Board.