IMMUNOLOGICAL CHARACTERIZATION OF ANTIGENS FROM *BACILLUS ANTHRACIS*

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ANTIGENS OF *B. ANTHRACIS*

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ABSTRACT

An antigen obtained from the filtrates of a modified casamino acid synthetic medium inoculated with the Sterne strain of B. anthracis, protected guinea pigs against virulent and lethal doses of B. anthracis and gave a zone of precipitation in capillary tubes and in gel diffusion tests in the presence of specific immune serum.

Gel diffusion and quantitative microprecipitation tests were employed for titrating anthrax antigen against its immune serum. The microprecipitation test was easier to run than the gel diffusion test and was more economical as it consumed less amount of immune serum.

An indirect hemagglutination test to detect anti-anthrax antibodies was developed during the course of these experiments. Human group O erythrocytes, tanned and sensitized with the culture filtrates containing the anthrax antigen, were used for titration of immune anthrax serum obtained from various sources. The indirect hemagglutination test was found to be more sensitive than the precipitation test.

Electrophoretic studies of B. anthracis culture filtrates showed a band in the gamma globulin region. No bands were seen when filtrates of growth medium were used instead.

Protein determinations of the anthrax antigen by chemical means were misleading because of the presence of large amounts of amino acids in the growth medium and because of the uptake of some of these amino acids by the organism during growth with possible production of new substances.

The protective activity of the anthrax antigen was destroyed by heat at 60°C for 1 hour, and by 0.5% trypsin. It was adsorbed on Seitz filter pads and was precipitated with alum. However, when the culture filtrate containing the antigen was heated, treated with trypsin and passed through Seitz filter pads, its precipitating properties were not lost. Alum precipitated antigen had no precipitating properties. From these findings it was postulated that at least two antigens were present in the culture filtrate of B. anthracis. The first of these was a protective antigen which was protein in nature, and the second a precipitating antigen of unknown chemical constitution, possibly a polysaccharide. Further work should be carried out to elucidate the exact chemical nature of the protective antigen and the precipitating antigen.

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INTRODUCTION

Studies in the past years have revealed that the capsule of Bacillus anthracis is a polypeptide made of D-glutamic acid and its cell wall contains a polysaccharide and a polyglycerophosphate. All three of these substances react with anthrax immune serum in vitro but none is protective as indicated in tests on laboratory animals.

The toxin produced by B. anthracis in vivo and in vitro in media containing amino acids, vitamins, inorganic salts and pyrimidines, has been fractionated into three components: Factor I (edematous Factor), Factor II (protective antigen) and Factor III (lethal Factor). Factor I, in combination with Factor II when inoculated into animals produces cutaneous edema. Factor III, when given in combination with Factor II, is lethal to animals. Factors II and III when administered separately, induce immunity in experimental animals. All three of these Factors are protein in nature and are antigenic. While both Factors II and III are protective to laboratory animals, interest has been mainly centered on Factor II because of its ease in production in artificial media, and its lack in toxic properties. In addition to protective tests in vivo, gel diffusion and complement fixation tests have also been employed to detect this antigen in vitro. There is little evidence to indicate that Factor II is super-

ior as an immunizing agent to the conventional spore vaccine, yet its easy availability makes it an attractive substance to utilize for measuring immunity in anthrax both *in vitro* and *in vivo*.

Considerable work dealing with the immunology of the protective antigen has been carried out by people working in this field, yet much work remains to be done in developing technics for identifying this antigen and detecting it by various serologic procedures. The experiments described in the present work were designed (1) to characterize the protective antigen by various chemical, physical and electrophoretic procedures, (2) to determine by a capillary precipitation technic the titer of the protective antigen in the culture filtrate and, (3) to measure quantitatively the antibody content of immune anthrax serum by the employment of an indirect hemagglutination test.

REVIEW OF THE LITERATURE

Six antigens of *Bacillus anthracis* have so far been identified and studied. These are the capsular polypeptide, the somatic polysaccharide, a polyglycerophosphate, and Factors I, II and III of the anthrax toxin.

Thorne (1960) classified *B. anthracis* into three major groups: (1) virulent strains (S type) which produce capsules when grown *in vivo* or *in vitro* in the presence of an added quantity of sodium bicarbonate and in an atmosphere rich in carbon dioxide, (2) avirulent strains which produce capsules in the absence of an added quantity of sodium bicarbonate or carbon dioxide, and (3) avirulent strains (R type) which do not produce capsules under any condition.

Studies on the chemical nature of the capsule by Thorne (1960) showed that it was composed of a polypeptide which was released upon autolysis or by autoclaving the cells. D-glutamic acid was the only compound released upon hydrolysis of the polypeptide. Keppie *et al.* (1963) showed that the capsule enhances virulence, is antiphagocytic and protects the organism from the bactericidal action of normal horse serum. Leonard and Thorne (1961) found that the glutamyl polypeptide reacted with *B. anthracis*, *Shigella flexneri*, *Pasteurella pestis* and *Salmonella typhosa* antisera as well as with normal sera, both in comple-

ment fixation and gel diffusion tests, and they thought that this non-specific reaction was due to serum lysozyme or to substances similar to it.

Smith and Strange (1956) studied the chemical nature of the polysaccharide of the cell wall. They showed that it consists of galactose 34%, galactosamine 34%, nitrogen 4.2%, and alpha-carboxylamino-nitrogen 0.8%. Attached to this polysaccharide was a small peptide moiety containing alpha-E-diaminopimelic acid. The polysaccharide reacts in high dilutions with anthrax immune serum and has common antigenic components with the polysaccharide of type 14 pneumococci and with partially hydrolyzed blood group A substance. The polysaccharide plays no role in virulence.

McCarty (1959) isolated a simple polymer of glycerophosphate from a number of gram-positive bacteria, such as the B-hemolytic streptococci, staphylococci and Bacillus anthracis. This substance reacted with immune serum prepared against various gram-positive organisms containing glycerophosphate in their cell walls.

It has long been known that B. anthracis produces a toxin which along with the capsule constitute the major factors concerned with virulence, but only recently this toxin has been fractionated into components and studied. Cromartie et al.

(1947a) were successful in preparing sterile extracts from anthrax lesions in rabbits, which when injected intracutaneously into rabbits produced histopathological changes similar to those produced by infection with B. anthracis. Repeated intracutaneous injections of this extract produced immunity in rabbits to anthrax infection.

Cromartie et al. (1947b) fractionated the extract from anthrax lesions using calcium phosphate. They showed that the precipitate contained Factors I and III, responsible for damaging tissue, and the supernatant contained Factor II, which had the immunizing property, and called it the protective antigen.

Stanley et al. (1960) and Stanley and Smith (1961) purified Factors I and II of the anthrax toxin, and isolated a third factor (Factor III) which was serologically different from Factors I and II, and was not toxic when injected alone, but lethal to mice and guinea pigs when given in combination with Factor II. Their work was confirmed by Beall et al. (1962) who isolated and purified the three factors. Beall et al. (1962) further showed that a combination of Factors II and III was lethal to mice and rats but caused no cutaneous edema in guinea pigs. The combination of Factors I and II was not lethal to animals but caused cutaneous edema in guinea pigs. Finally they believed that the lethal effect of the

toxin is best measured on rats.

While work was being carried out to identify various toxic and antigenic factors in edema fluid, attempts were also made to produce similar substances *in vitro*. Gladstone (1946) was successful in preparing the protective antigen (Factor II) by growing *B. anthracis* in serum or plasma. Sintered glass filtrates of such cultures immunized rabbits against a virulent strain of *B. anthracis*. Various semi-synthetic and chemically defined media were used to produce this protective antigen. For example, Belton and Strange (1954a) prepared a casamino acid-synthetic medium which was later modified by Thorne and Belton (1957). Likewise, Wright *et al.* (1954b) prepared the 528 medium which he and his co-workers later (1954, 1957, 1962) modified. These media were designated as 555, 599, 687, 968 and 1095 (Table 1). In all these media a protective antigen was elaborated when inoculated with *B. anthracis*.

Smith *et al.* (1958) were successful in producing a toxin from *B. anthracis* by growing the organism in a medium containing 90% serum and they concluded that serum was necessary for toxin production. Later, Strange and Thorne (1958) indicated that enzymes produced in the cultures during growth destroyed the toxin. By adding serum or sodium bicarbonate to bring up the pH to 8-9, the enzyme action was inhibited and

the toxin could be detected. Puziss and Howard (1963) showed that sodium bicarbonate also altered the permeability of the bacterial cell in such a way as to permit the protective antigen to diffuse out. In its absence the protective antigen accumulated in the cell. Thorne (1960) pointed out that Factors I and III were adsorbed by sintered glass filters and in order to prevent adsorption, 10% serum should be added to the culture prior to filtration. The serum blocks the adsorbing capacity of the glass filter and permits the passage of Factors I and III along with Factor II.

Various strains of B. anthracis have been tried for their ability to produce a protective antigen in culture media. Wright et al. (1962), isolated six NP and R mutant strains from six typical virulent strains of B. anthracis, and compared their ability to produce a protective antigen in different media with that of the Sterne strain. The isolated mutants were V770-NP1-R_1, 1062-NP1-R_3, 107-NP2-R_2, 108-NP1-R_2, 116-NP1-R_2 and 1133-NP1-R_3. The Sterne strain was superior to these mutants in the elaboration of the antigen in the casamino acid synthetic medium and 528, 555, 599 and 687 media. However, when the 968 and the 1095 media were used instead and the incubation period was prolonged to 40 hours, the isolated mutants, especially the V770-NP1-R_1 and 107-NP2-R_2, were found to be superior in elaborating the antigen as compared with the Sterne strain.

Gladstone (1946) showed that rabbits, sheep and monkeys immunized with the protective antigen survived the challenge of a virulent strain of B. anthracis. Guinea pigs immunized with the antigen gave a prolonged survival time as compared with controls, but no immunity was produced in mice inoculated with the antigen. McGann et al. (1961) showed that the prolongation of life of test guinea pigs treated with the antigen was statistically valid and could be used as a criterion for evaluating the protective capacity of the antigen.

Serological studies have also been carried out to characterize the protective antigen. For example, Gladstone (1946) and Cromartie et al. (1947b) were unable to obtain precipitation using anthrax immune rabbit serum and the protective antigen. Thorne and Belton (1957) however, were successful in obtaining bands of precipitation in the gel diffusion technics using horse antiserum instead. McGann et al. (1961) developed a complement fixation test using the protective antigen and rabbit antisera prepared by immunizing rabbits with the same antigen. However, fixation of the complement was not obtained when rabbit antiserum, prepared by immunizing rabbits with living organisms, was used in the test.

Gladstone (1946) showed that the protective antigen was not dialyzable but was destroyed at pH below 6.5, by pas-

teurization and trypsin digestion (Cromartie et al., 1947b). The protective antigen could be precipitated by alum (Wright et al., 1954a) and by trichoroacetic acid and ammonium sulfate (Belton and Strange, 1954b). Belton and Strange (1954a) further showed that the antigen was adsorbed on Seitz filters, and its activity was best preserved when stored at -20°C, or at room temperature in lyophilized form. From the above it was concluded that the antigen was protein in nature.

Klein et al. (1962) showed that the protective antigen when inoculated into guinea pigs produced a level of resistance approximately 1000-fold higher than in the controls, and when a combination of protective antigen and live vaccine was given, this resistance was 100 million-fold. Live vaccines alone produced only a 10-15-fold resistance as compared with the controls.

MATERIALS AND METHODS

Bacillus anthracis Strains:

A local virulent strain, isolated from a horse with anthrax*, was used to challenge animals, and the Sterne strain, an avirulent and uncapsulated strain obtained from the National Collection Type Cultures, Collindale, was used to elaborate the protective antigen. Both strains were used as spore suspensions.. Spores were prepared by streaking the organisms on tryptose phosphate agar plates and incubating them at 37°C for three days. By this time most of the organisms had sporulated. The growth was suspended in 0.14 M sodium chloride solution (physiological saline), washed three times with the same solution, centrifuged and the sediment resuspended in sterile distilled water. The spore suspensions were then heated at 60°C for 90 minutes to kill any vegetative forms.

Three spore suspensions were prepared. The first two of these suspensions were prepared from the Sterne strain and contained 10×10^6 and 3×10^9 spores/ml. respectively. The third, prepared from the virulent strain, contained 100×10^6 spores/ml. Spore counts were performed by the plate dilution method. The suspensions were stored at 4°C until used.

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*Isolated in 1955 by Dr. Joseph Asmar, School of Agriculture, American University of Beirut.

Preparation of Protective Antigen

The protective antigen was prepared using the modified casamino acid synthetic media described by Thorne and Belton (1957). Five stock solutions were prepared:

Solution A

Casamino acids (Difco)	90.00 grams
Glycine	00.375 "
L-tryptophan	01.30 "
L-cystine	00.30 "

L-tryptophan was dissolved in 10 ml. of 1N HCl. L-cystine was dissolved in 3 ml. of distilled water, and concentrated HCl was added dropwise until it was completely dissolved. These solutions were then added to the casamino acid and glycine dissolved in distilled water, and the volume was made up to one liter with distilled water.

Solution B

Calcium chloride ($CaCl_2.6H_2O$)	0.5512 grams
Magnesium chloride ($MgSO_4.7H_2O$)	0.25 "
Adenine	0.07 "
Guanine	0.075 "
Uracil	0.070 "
Thiamine	0.0025 "

Guanine was dissolved in distilled water and concentrated HCl was added dropwise until it was completely dissolved. It was then added to the rest of the ingredients dissolved in distilled water and the volume was made up to one liter with distilled water.

Solution C

Potassium dihydrogen phosphate	34.00 grams
Potassium hydroxide 5%	160 ml.

Potassium dihydrogen phosphate was dissolved in 800 ml. of distilled water to which 160 ml. of 5% potassium hydroxide was then added and the volume was made up to one liter with distilled water.

Solution D

Sodium bicarbonate	60.00 grams
D-glucose	10.00 "
Glutamine	00.01 "

These were dissolved in 900 ml. of distilled water. This stock solution was sterilized by filtration using sintered glass.

Solution E

Ferrous sulfate ($FeSO_4.7H_2O$)	1.40 grams

Manganese sulfate ($MnSO_4 \cdot 4H_2O$) ..... 0.0223 grams

Ferrous sulfate was dissolved in 200 ml. of distilled water containing 0.3 ml. of concentrated HCl and was then mixed with the manganese sulfate dissolved in distilled water. The volume was made up to 400 ml. with distilled water. It was then filtered through sintered glass. To this solution, 0.1 ml. of a 10×10^6 spores/ml. suspension of the Sterne strain was added.

The above stock solutions were stored at 4°C when not in use. Solutions D and E were used within 72 hours of preparation.

Twenty milliliters from each of stock solutions A, B and C were added to 385 ml. of distilled water in a 1000 ml. flask. This mixture was then autoclaved for 15 minutes at 121°C. Next, 45 ml. of solution D and 10 ml. of solution E containing spores, were added aseptically. The culture was then incubated at 37°C for 24 hours. Development of turbidity in the culture medium and presence of large Gram-positive rods in stained smears were considered as criteria for growth. The culture was then filtered through sintered glass. The filtrate was tested for the presence of antigen. The sterility of the filtrate was tested by streaking a tryptose-phosphate agar plate with a sample of the filtrate and incubating

it at 37°C for 48 hours. Fifty milliliter screw-cap tubes were filled each with 20 ml. of culture filtrate and were stored at -20°C for further use.

Medium for control purposes was prepared following the same procedure as described for the preparation of the antigen, except that no spore innoculum was added to it.

Preparation of Immune Serum

The inoculum used for immunizing rabbits was a suspension of the Sterne strain containing 3×10^9 spores/ml. Three rabbits were immunized with this suspension using the following schedule.

Amount injected/rabbit	Route	Time of injection
0.1 ml.	Intracutaneously	1st week
0.2 ml.	Subcutaneously	2nd week
0.4 ml.	"	"
0.6 ml.	"	3rd week
0.8 ml.	"	"
1.0 ml.	"	"

Seven days after the last immunizing dose, each rabbit was inoculated with 0.5 ml. of the virulent strain containing 100×10^6 spores/ml. The immune animals resisted this challenge dose while the controls died. Ten days after the last inocula-

tion the rabbits were bled from the heart and the serum was separated and stored at -20°C until used.

Anti-anthrax serum prepared in horses (used for the Ascoli test) was obtained from the Pasteur Institute, Paris, and anti-anthrax serum prepared in cattle was obtained from Berna, Switzerland.

Protective Antigen Concentration

1. Lyophilization: Neutral glass freeze-drying ampoules were filled each with 2.5 ml. of the culture filtrate (containing the protective antigen) and were lyophilized*. Growth medium was also lyophilized in the same way to be used as control. These preparations were used for protein determinations and electrophoresis studies.

The culture filtrate and the growth medium were also lyophilized using the procedure of Thorne and Belton (1957). These were used in gel diffusion and protection tests. The material to be lyophilized was made 10% V/V with a 2% W/V gelatin solution in 0.02 M phosphate buffer, pH 8. All lyophilized preparations were stored at room temperature in a dissicator when not in use.

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*"Speedivac" Centrifugal Dryers - Model L5, Edwards High Vacuum Ltd., Sussex, England.

2. Precipitation of the Antigen: The method of Belton and Strange (1954a) was followed. Alum, 0.1 gram was added to 100 ml. of culture filtrate and the pH was adjusted to 5.9 with 1N HCl. This was left overnight at 4°C after which the supernatant was removed and the precipitate suspended in 100 ml. of distilled water. A portion of the medium for control purposes was treated in a like manner. Both suspensions were used in protection tests. For hemagglutination tests the precipitate formed was dissolved in 5 ml. of 0.2 M citric acid and the volume made up to 75 ml. with physiological saline. The solution was then dialyzed against distilled water at 4°C for 48 hours.

All precipitated preparations were stored at 4°C until used.

3. Ultrafilter: The ultrafilter* used in these tests consisted of a nylon frame covered with a sac of dialysis tubing. Attached to the nylon frame was a rubber tubing extending to a vacuum pump. The ultrafilter was immersed into a solution containing the protective antigen and suction was applied. With such a preparation water and solutes of molecular weight below 30,000 would pass through the dialysis tubing, while large molecular weight compounds, such as

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*Obtained from LKB-Produkter AB, P.O.B. 12220, Stockholm 12, Sweden.

proteins, would not. Both the culture filtrate and the control medium were concentrated 4-fold by this method. These concentrated forms were used in the indirect hemagglutination tests.

4. Concentration by "Aquacide": Culture filtrate and growth medium were placed in dialysis bags and immersed in "Aquacide"*. At 15-minute intervals the "Aquacide" adhering to the dialysis bags was wiped off. By this procedure up to 8-fold concentrations were obtained over a period of 8 hours. These preparations were used in the indirect hemagglutination tests.

Protective Tests

Four groups of animals, each group consisting of 4 locally purchased guinea pigs weighing approximately 350 grams each, were used in these tests.

The first group of animals was immunized according to the method of McCann et al. (1961). The contents of 6 lyophilized tubes containing the antigen were dissolved in 3 ml. of distilled water. Of this solution, 0.5 ml. was injected subcutaneously into each guinea pig in the group. This was

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*"Aquacide" is a resin obtained from Calbiochem., 3625 Medford Street, Los Angeles, California.

repeated on alternate days until each guinea pig in the group had received a total of 2.5 ml., equivalent to 12.5 ml. of untreated culture filtrate. A second group was immunized using an alum-precipitated antigen (Belton and Strange, 1954a). Alum-precipitated suspension obtained from the culture filtrate containing the antigen was injected in 1.5 ml. amounts subcutaneously into each guinea pig in the group. Ten days later another 1.5 ml. dose was given to each guinea pig. Total amount of alum-precipitated suspension injected to each guinea pig was equivalent to 3 ml. of untreated culture filtrate.

A third and a fourth group of guinea pigs were inoculated in a like manner using lyophilized growth medium and alum-precipitated growth medium respectively. These animals served as controls in protection tests.

Seven days after the last immunizing injection, each guinea pig was challenged with 0.5 ml. of the virulent spore suspension containing 100×10^6 spores/ml. (Table III).

Gel Diffusion Tests

The method of Thorne and Belton (1957) was used for these tests. The medium used consisted of phosphate buffer, 0.075 M, (pH 7.3) containing 0.9% sodium chloride, 0.01% merthiolate and 1% agar. Petri dishes with a diameter of

9 cm. were filled each with 12 ml. of medium and were stored at 2°C until used. Two rows of wells were bored out in the agar in such a way that each well of one row was exactly facing its corresponding well in the other row at a distance of 0.5-1 cm. Horse antiserum was placed in the wells of the first row, and the plate was stored at 4°C for 24 hours. The contents of 6 lyophilized tubes containing the antigen were reconstituted by the addition of 15 ml. of physiological saline and serial dilutions were prepared from this. Next, the wells of the second row were filled each with a different dilution of this antigen. The plate was left at room temperature for 24 hours after which bands of precipitation between the immune serum and different dilutions of antigen were observed. A similar procedure was carried out using undiluted antigen and serial dilutions of horse immune serum (Fig. 1 and Table IV).

Three other gel diffusion plates were prepared in such a way that 3 rows of wells, each row consisting of 2 wells facing each other, were bored out. Horse antiserum was placed in one of the wells in the middle row, and normal horse serum in the other well facing it. The plate was stored at 4°C for 24 hours after which undiluted antigen was placed in the two empty wells of one row and undiluted control medium in the two other empty wells of the other row. The plate was left at room temperature for 24 hours after which they were ob-

served for bands of precipitation.

A similar procedure was carried out for testing immune rabbit and bovine sera. Samples of protective antigen treated with 0.5% trypsin at 37°C for 1 hour, heated at 60°C for 1 hour, autoclaved and passed through Seitz filter, were tested successively in a like manner.

Capillary Tube Precipitation Test

Pasteur pipets about 5 cm. long were prepared from soda lime glass tubing and were used in this test. Serial dilutions of culture filtrate were prepared. The Pasteur pipets were sealed on one end by sticking them into clay. They were then half filled with horse antiserum and were layered with different dilutions of the culture filtrate. The readings were taken after 30 minutes. Similar procedures were carried out for testing normal horse serum, immune rabbit serum, normal rabbit serum, immune bovine serum and normal bovine serum. Likewise, tests were carried out using control medium with various test sera in place of the culture filtrate (Table V).

Immune serum was also titrated by using a quantitative micro-precipitation technic in capillary tubes. This was done by placing different dilutions of antisera in capillary tubes and overlaying them with undiluted antigen.

Indirect Hemagglutination

1. Sensitizing the Erythrocytes: The method of Garabedian et al. (1957) was followed with some modifications. Fresh, human, group O, Rh positive or negative blood, 20 ml., was mixed with 20 ml. of Alsever's solution. This was stored at 4°C and was used within 7 days after storage. A portion of the blood was centrifuged and the erythrocytes were then washed with 10 ml. of physiological saline. This was repeated 3 times. Packed erythrocytes, 0.5 ml. were suspended in 9.5 ml. of 1:20,000 tannic acid solution in physiological saline. The mixture was incubated at 37°C for 10 minutes and was frequently shaken. It was then centrifuged. The cells were then washed with 10 ml. of physiological saline, and were suspended in 9.5 ml. of sensitizing antigen. Next, the mixture was left at room temperature for 25 minutes, shaking it occasionally. It was then centrifuged, the supernatant removed, and the cells washed once with 10 ml. of 2% bovine albumin in physiological saline. For the test a 0.5% suspension of sensitized cells were prepared in 2% bovine albumin.

Red blood cells were sensitized successively with the antigen and growth medium in their original concentration and at 2-fold, and 4-fold concentrations. They were also sensitized with alum precipitated antigen dissolved in 0.2 M citric acid.

Normal and tanned erythrocyte suspensions for control purposes were prepared by suspending 0.5 ml. of washed packed erythrocytes in 100 ml. of 2% bovine albumin in physiological saline.

2. Antiserum Titration: In a rack 17 round-bottomed test tubes (100x13 mm) were arranged. To the first tube of this series 0.9 ml. of physiological saline and 0.1 ml. of the serum sample to be tested, and to the rest of the tubes, excluding the seventeenth tube, 0.25 ml. of 2% bovine albumin in physiological saline were added. The first tube, containing 1:10 dilution of the test serum was then removed from the rack and placed in a water bath at 60°C for 20 minutes. After this time 0.75 ml. of diluted serum was removed, 0.25 ml. of it was added to the second tube, 0.25 ml. to the seventeenth tube and the rest, 0.25 ml., was discarded. The contents of the second tube were mixed and 0.25 ml. of the fluid was carried to the third tube. This process was repeated until the fifteenth tube was reached. From the fifteenth tube 0.25 ml. of the fluid was discarded. Next 0.5 ml. of sensitized erythrocytes was added to each of the first 16 tubes. The seventeenth tube received the same amount of 0.5% normal erythrocytes. The tubes were left at room temperature for 1-2 hours aft which the readings were taken according to the pattern of settled erythrocytes (Table II and VI).

Tests were carried out on horse, bovine, and rabbit anti-anthrax sera and normal sera, and erythrocytes sensitized with various types of antigen and growth medium were used in each case.

Protein Determination

Gelatin-free lyophilized antigen, (equivalent to 15 ml.), was dissolved in 3 ml. of physiological saline and its protein content was determined by the Biuret method (Cornall et al., 1949). The protein content of the growth medium was likewise determined.

Electrophoresis

One package of B-2 buffer (Veronal buffer, containing 2.76 grams of diethyl barbituric acid and 15.4 grams of sodium diethyl barbiturate, Spinco) was dissolved in 1 liter of distilled water. Of this buffer solution 800 ml. was placed in the electrophoretic cell vessel (Spinco model R type) and 8 paper strips (Schleicher and Schnell 2043 A mgl, 3 cm. wide and 30.6 cm. long) were attached and soaked in the buffer. Gelatin-free lyophilized antigen was dissolved in enough distilled water to give a 15-fold concentration of the original antigen. A similar procedure was carried out for the growth medium. With an applicator, two paper strips were streaked with the 15-fold concentrated antigen, two with the 15-fold concentrated growth medium and two others with normal rabbit

serum. The current (Spinco duostat) was then turned on and was left on for 16 hours. The paper strips were then removed and placed in an oven at 121°C for 30 minutes, in methanol for 6 minutes, B-4 dye (1 gram of bromophenol blue dissolved in 1 liter of methanol, Spinco) for 30 minutes, and in 3 solutions of 5% acetic acid each for 6 minutes. They were then dried in an oven at 121°C for 15 minutes. Finally, they were exposed to ammonia fumes for 15 minutes and were scanned with an analytrol (Spinco model RB) (Fig. 2 and 3).

TABLE I

Media that elaborate the protective antigen

Ingredient	528 Molar conc.	555 Molar conc.	599 Molar conc.	687 Molar conc.	968 Molar conc.	1095 (for anaerobic growth) Molar conc.
glycine	0.0004	-	-	-	0.0003	0.0003
DL-alanine	0.001	-	-	-	0.0001	0.0001
DL-serine	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002
DL-threonine	0.001	0.0005	0.0005	0.0005	0.0005	0.0005
DL-valine	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
DL-leucine	0.001	0.001	0.001	0.001	0.001	0.001
DL-isoleucine	0.001	0.001	0.001	0.001	0.001	0.001
DL-aspartic acid	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
L-glutamic acid	0.0011	0.0011	0.0011	0.0011	0.0011	0.0011
DL-lysine.HCl	0.00016	-	-	-	-	-
L-arginine.HCl	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
L-histidine.HCl	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
L-cystine	0.00005	-	-	-	-	-
L-cysteine.HCl	-	0.00005	0.00005	-	-	-
DL-methionine	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002
L-proline	0.001	0.00025	0.00025	0.00025	0.00025	0.00025
DL-phenylalanine	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004
L-tryptophan	0.00025	0.00025	0.00025	0.00025	0.00025	0.00025
L-glutamine	0.000007	-	-	-	-	-
$Ca.Cl_2.6H_2O$	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
$Mg.SO_4.7H_2O$	0.00004	0.00004	0.00004	0.00004	0.00004	0.00004
$Mn.SO_4.4H_2O$	0.000005	0.000005	0.000005	0.000005	0.000005	0.000005
NaH_2PO_4	0.005	0.005	0.005	0.005	0.005	0.005
K_2HPO_4	0.005	0.005	0.005	0.005	0.005	0.005
$Na.HCO_3$	0.03	0.03	0.03	0.03	0.03	0.03
adenine	0.00002	0.00002	-	-	-	-
guanine.HCl	0.00002	0.000014	0.000014	-	0.000045	0.000045
uracil	0.000025	0.000025	-	-	-	-
xanthine	-	-	-	0.00004	-	-
thiamine.HCl	0.0000003	0.0000003	0.0000003	0.0000003	0.0000003	0.4 ug/cc
glucose	0.0056	0.0056	0.0056	0.0056	0.0056	0.0056
adenosine	-	-	-	-	1 ug/cc	1 ug/cc
biotin	-	-	-	-	-	0.4 ug/cc
pyridoxal.HCl	-	-	-	-	-	1 ug/cc

Inoculum: The inoculum used in each case were spores of B. anthracis.

Modified 599: This differed from 599 medium by having an extra 0.002 M glycine and 0.005 M DL-alanine. The amount of aspartic acid was increased to 0.0025 M, glutamic acid to 0.0055 M and sodium bicarbonate to 0.072 M; $FeSO_4.7H_2O$ was decreased to 0.000025 M. The vegetative form of the Sterne strain was used instead of spores.

Casamino acid synthetic medium: This differed from the modified casamino acid synthetic medium (page 11-13) in having a reduced amount of sodium bicarbonate (25 grams in stock solution D) and an increased amount of ferrous sulfate (2.78 grams in stock solution E).

TABLE II

Procedure of the indirect hemagglutination test for anthrax

Test* serum	Dilution**	Test							Controls	
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	Sera. erythro-cytes	Test serum (1:10)
	ml.	0.25	0.25	0.25	0.25	0.25	0.25	0.25	-	0.25
2% bovine albumin in saline. ml.		-	-	-	-	-	-	-	0.25	-
Sensitized*** human group O, Rh negative erythrocytes (0.5%). ml.		0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	-
Normal human group O, Rh negative erythro-cytes (0.5%). ml.		-	-	-	-	-	-	-	-	0.25

*Sera tested were horse, bovine, and rabbit anti-anthrax sera and normal sera.

**Except for dilution 1/10, the other dilutions of serum were made in 2% bovine albumin. Serum dilutions up to 1/5,242,800 were carried out, but because of lack of space, these dilutions were not indicated in this Table.

***Erythrocytes were sensitized with anthrax antigen and with growth medium as control.

RESULTS

The results presented in Table III indicate that 50% of the guinea pigs immunized with the lyophilized anthrax antigen, and 25% of the guinea pigs immunized with alum-precipitated antigen survived the virulent challenge dose. The rest of the immunized guinea pigs survived longer than the controls.

The results of gel diffusion tests are presented in Table IV. Two bands of precipitation one thick and the other thin, were obtained in the gel diffusion tests using the antigen and immune horse serum (Fig. 1). When immune bovine serum was used instead of immune horse serum only a thin band of precipitation appeared at the equivalence zone. No band of precipitation was obtained when immune horse serum was replaced by immune rabbit serum, normal rabbit serum, normal horse serum, and normal bovine serum. Likewise, when the antigen was replaced by growth medium no band of precipitation appeared. A gel diffusion titer of 1/8 was obtained by titrating the antigen with undiluted immune horse serum, but no band of precipitation appeared when different dilutions of immune horse serum were used with undiluted antigen.

It can be seen from Table V that the antigen as well as the growth medium showed a band of precipitation with

undiluted immune horse serum in the microprecipitation tests. However, when 1/5 dilution of immune horse serum was used instead of undiluted serum, the positive results obtained with the growth medium were eliminated. Titration of 4-fold concentrated antigen by the microprecipitation technic gave a titer of 1/128 but no band of precipitation appeared with unconcentrated antigen or with 1/2 dilution of the antigen. A titer of 1/20 was obtained when immune serum was used with undiluted antigen in microprecipitation tests. The results were negative with normal horse, bovine and rabbit sera, and with immune bovine and rabbit sera.

The activity of the precipitating antigen, as indicated by the gel diffusion tests (Fig. 1) and the microprecipitation technics, was not destroyed when the antigen was heated at 37°C for 1 hour, 60°C for 1 hour, autoclaved, or passed through a Seitz filter pad. Likewise it was not destroyed by 0.5% trypsin at 37°C for 1 hour. However, negative results were obtained when alum precipitated antigen, dissolved in 0.2 M citric acid, was used in gel diffusion tests.

As indicated in Table VI a titer of 1/1280 for immune rabbit serum, 1/10,240 for horse and $\geq$1/5,242,800 for bovine immune serum, were obtained using untreated protective antigen in indirect hemagglutination tests. When 2-fold concen-

trated antigen was used, the immune rabbit serum titer was 1/2560. Inconsistent results were obtained when 4-fold concentrated antigen was used for sensitizing the erythrocytes.

The protein content of the 5-fold concentrated culture filtrate containing the antigen was 0.75 mg/ml., while the protein content of the 5-fold concentrated growth medium was 0.60 mg/ml. The electrophoretic pattern of the culture filtrate showed a peak in the gamma globulin region. No peak appeared when growth medium was used for such determinations (Fig. 2 and 3).

TABLE III

Protective activity of the anthrax antigen in guinea pigs

Immunizing material	Total number of animals used	Day of death following challenge with a virulent strain of *B. anthracis**							Survivors
		1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	
Alum precipitated antigen	4	-	-	-	1	-	2	-	1
Lyophilized antigen	4	-	-	-	-	1	1	-	2
Lyophilized antigen	4	-	-	-	-	-	1	1	2
Alum precipitated growth medium (control)	4	-	4	-	-	-	-	-	-
Lyophilized growth medium (control)	4	-	-	4	-	-	-	-	-

*7 days after the last immunizing injection each guinea pig was challenged with 0.5 ml. of a virulent strain suspension containing 100×10^6 spores/ml.

TABLE IV

Gel diffusion titrations of the anthrax antigen with anthrax immune sera

Type of serum	Type of material tested							
	Growth medium (control)	Culture filtrate (*B. anthracis*) dilutions:					Alum ppt. antigen dissolved in 0.2 M citric acid	Alum ppt. growth medium dissolved in 0.2 M citric acid (control)
		1/1	1/2	1/4	1/8	1/16		
Immune horse serum	-	+++	+++	++	++	-	-	-
Normal horse serum	-	-	-	-	-	-	-	-
Immune bovine serum	-	+	-					
Normal bovine serum	-	-						
Immune rabbit serum	-	-						
Normal rabbit serum	-	-						

+++ = Two bands (one thick and one thin) of precipitation

++ = One band (thick) of precipitation

+ = One band (thin) of precipitation

- = Precipitation absent

TABLE V

Quantitative microprecipitation tests using anthrax antigen and anthrax immune sera

Type of serum	Serum dilutions	Growth medium undiluted (control)	Antigen dilutions				
			undiluted	1/2	1/4	1/8	1/16
Immune horse serum	undiluted	+	+	+	+	+	+
	1/5	-	+	+	+	+	-
	1/10	-	+				
	1/20	-	+				
	1/40	-	-				
Normal horse serum	undiluted	-	-				
Normal bovine serum	"	-	-				
Immune bovine serum	"	-	-				
Normal rabbit serum	"	-	-				
Immune rabbit serum	"	-	-				

+ = Precipitation present

- = Precipitation absent

TABLE VI

Indirect hemagglutination test using anthrax antigen and anthrax immune sera

Sensitizing substance		Antiserum titer			Normal serum
		Rabbit	Horse	Bovine	Rabbit Horse Bovine
Anthrax antigen concentration:	1-fold	1/1280	1/10240	>1/5,242,800	Negative
	2-fold	1/2560	N.D.	N.D.	Negative
	4-fold	Inconsistent results	Inconsistent results	Inconsistent results	Negative
Alum ppt. antigen dissolved in 0.2 M citric acid		Negative	N.D.	N.D.	Negative
Growth medium 1-fold, 2-fold and 4-fold concentrations		Negative	Negative	Negative	Negative

N.D. = Not done

DISCUSSION

During the course of the experiments described herein it was found that the quantitative microprecipitation test was a more convenient method for titrating the anthrax antigen and/or the antiserum than the gel diffusion test, and the indirect hemagglutination test for titrating anthrax immune serum was more sensitive than the precipitation test. It was further observed that the electrophoretic pattern of the anthrax antigen pointed out to a band in the gamma globulin region, and the chemical and physical agents which destroyed the protective activity of the anthrax antigen, did not effect its precipitating properties.

Attempts to prepare the anthrax antigen in 599, 698 and 1095 media (Wright et al., 1954b, 1962 and Wright and Puziss, 1954, 1957) using spores of Sterne strain as inoculum, were unsuccessful. In these media no growth occurred following incubation at 37°C. Wright et al. (1962) indicated that serial transfers were necessary for the strain to adopt itself to the growth medium, and this was probably the reason for the absence of growth in the above mentioned media.

Thorne and Belton (1957) prepared a modified casamino acid synthetic medium and a modified 599 medium for the propagation of B. anthracis. The inoculum used in the casa-

mino acid medium was spores of the Sterne strain. Growth in this medium did occur possibly because this was a more enriched medium than the 599, 698 and 1095. The inoculum used in the modified 599 medium was the vegetative form of the Sterne strain. In our experiments it was found that gel diffusion titers, obtained by using the antigen prepared in a modified casamino acid synthetic medium, were higher than those obtained using the modified 599 medium.

McGann et al. (1961) developed a method for demonstrating the presence of anthrax antigen in culture filtrates by showing its protective activity in guinea pigs. In 5 of the groups of animals there was a prolongation of life with no survivals, in 4 groups 25% survived, in 2 groups 50% survived, in 1 group 75% survived, and in the last group 100% survived. They then showed that the prolongation of life, and the number of survivals were statistically valid and could be used as criterion for demonstrating the presence of anthrax protective antigen in culture filtrates. In our experiments results similar to those of McGann were obtained when anthrax antigen from modified casamino acid medium was tested in groups of guinea pigs.

Further indication that the anthrax protective antigen was present in the culture filtrate of casamino acid medium was obtained by the utilization of the gel diffusion

test described by Thorne and Belton (1957). However, experiments carried out in this laboratory indicated that a positive gel diffusion reaction did not necessarily mean that the reacting antigen was protective.

The quantitative microprecipitation test and the indirect hemagglutination test, both of which were used in this studies to titrate the anthrax antigen and/or antiserum, were found to be more convenient and more sensitive than the gel diffusion test. Performance of the gel diffusion test and observation of results required 3 days, whereas, in the microprecipitin test, it took 30 minutes to obtain the results. Furthermore, less antiserum was consumed in the microprecipitin test than in the gel diffusion test. Likewise, the indirect hemagglutination test was found to be more sensitive than the precipitation tests. For example, while immune rabbit serum failed to give positive results in the precipitation tests with anthrax antigen, it yielded a high titer in hemagglutination tests.

The electrophoretic analysis of the culture filtrate and the growth medium was so far found to be the best method to indicate the presence of protein in the culture filtrate and its absence in the growth medium. When B. anthracis culture filtrates were used a band appeared in the gamma globulin region, whereas no bands were seen when filtrates of

growth medium were used instead (Fig. 2). Protein determination of the anthrax antigen by chemical means were misleading because of the presence of large quantities of amino acids in the growth medium and because of the uptake of some of these amino acids by the organism during growth with possible production of new substances.

Gladstone (1946) and Cromartie et al. (1947b) indicated that the protective activity of the antigen was lost when it was heated at 60°C for 1 hour or treated with trypsin, suggesting that the antigen was protein in nature. Belton and Strange (1954a) showed that the protective antigen was adsorbed on Seitz filter pads and Wright et al. (1954a) pointed out that the protective antigen could be precipitated by the addition of alum. Tests performed in this laboratory indicated that the precipitating activity of the antigen was not destroyed by heating at 60°C for 1 hour or by autoclaving at 15 lbs pressure for 15 minutes. Furthermore, gel diffusion titers of unheated antigen and antigen heated at 100°C for 5 minutes were identical. Bands of precipitation were obtained with culture filtrate (containing the antigen) that had been passed through Seitz filter pad and also with culture filtrate treated with trypsin. However, bands of precipitation were not obtained in gel diffusion and hemagglutination tests when alum precipitated antigen dissolved in 0.2 M citric acid was used. Nevertheless, in accordance with the findings of

previous investigators, the alum precipitated antigen was found to be protective in guinea pigs. The electrophoretic pattern of the anthrax antigen which had been heated at 100°C for 5 minutes showed a thin band at the point of application but did not give any peak when scanned with the analytrol.

On the basis of these findings and those of previous investigators it was postulated that at least two types of antigens existed in the culture filtrate. The first of these was a protective antigen, possibly a protein, which was destroyed by heat and trypsin, was adsorbed on the Seitz filter pad, precipitated by treatment with alum, but was inactive in gel diffusion tests. The second antigen was a precipitating antigen complex which was not destroyed by heat or by 0.5% trypsin, passed through Seitz filter pad, and was not precipitated by alum. The chemical nature of the precipitating antigen could not be determined at this time. It may have been a polysaccharide with the same or similar antigenic constituents as the antigen which gives the precipitation reaction in the Ascoli test (Ascoli, 1911). Further work should be carried out to elucidate the exact chemical nature of both the protective and the precipitating antigens.

Fig. 1. Gel diffusion precipitation of the anthrax antigen with anthrax immune sera. AD - antigen dilut' IHS - immune horse serum; NHS - normal horse serum; IBS - immune bovine serum; NBS - normal bovine serum; IRS - immune rabbit serum; NRS - normal rabbit serum; A - antigen; GM - growth medium (control); A_1 - untreated antigen; A_2 - antigen heated at 37°C for 1 hour; A_3 - antigen heated at 60°C for 1 hour; A_4 - autoclaved antigen; A_5 - antigen passed through Seitz filter pad; A_6 - antigen treated with 0.5% trypsin at 37°C for 1 hour.

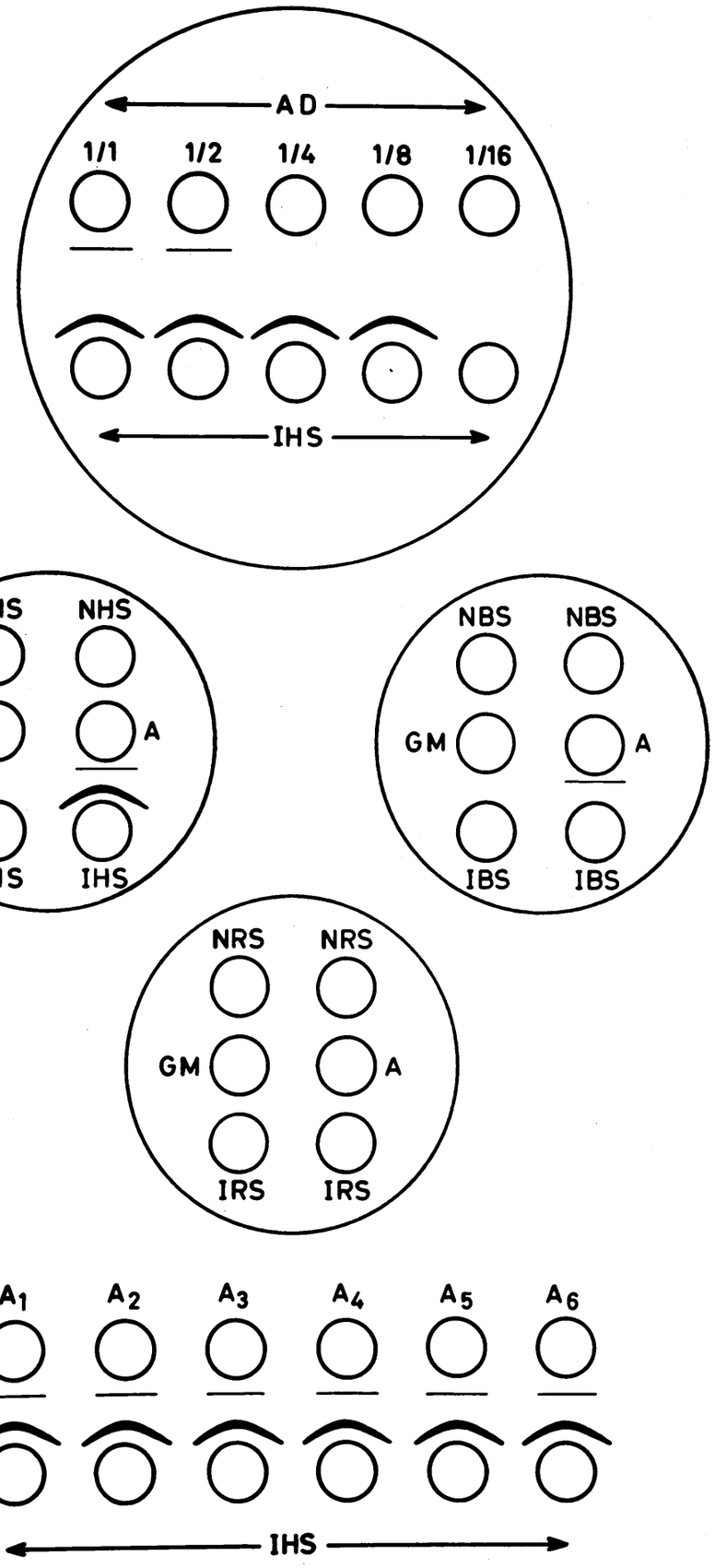

Fig. 2. Electrophoretic pattern of anthrax antigen, growth medium (control), and normal rabbit serum. [10 λ (0.01 ml.) was used in each case and a 600 mu filter was used in the analytrol for scanning].

Fig. 3. Electrophoretic pattern of anthrax antigen, growth medium (control), and normal rabbit serum. /10 λ (0.01 ml.) was used in each case and a 500 mu filter was used in the analytrol for scanning/.

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