STUDIES ON A PROTECTIVE ANTIGEN PRODUCED *IN VITRO* FROM *BACILLUS ANTHRACIS*: PURIFICATION AND CHEMISTRY OF THE ANTIGEN.

R. E. STRANGE AND F. C. BELTON.

From the Microbiological Research Department, Ministry of Supply, Porton, Wilts.

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ANTIGENS of *Bacillus anthracis* have been described by various workers over a number of years and two of them are chemically well defined. They are a capsular antigen (Tomcsik and Szongott, 1933), polyglutamyl peptide (Ivanovics and Erdos, 1937), and a somatic antigen, a polysaccharide (Ivanovics, 1938; 1940). Both these substances give precipitation reactions with the appropriate antiserum but neither is the protective antigen and it is evident that some other substance has this function. Gladstone (1950) stated that the protective antigen was protein in nature but he was unable to separate it by electrophoresis from the other protein present in his medium. In the preceding paper (Belton and Strange, 1954) details are given of the production of protective antigen in non-protein media based on the work of Wright, Hedberg and Slein (1954), and such substrates provide a more satisfactory basis for its isolation and study.

This paper records a chemical study of the non-dialysable products of bacterial growth (and possibly lysis) of strains of *B. anthracis* in culture filtrates of the media described. Details of the method of isolation of a protein, small amounts of which immunise rabbits against anthrax infection, and a polysaccharide similar to that reported by Ivanovics (1940), are given with the results of chemical analysis. Isolation of antigen from alum precipitates of culture filtrate is described.

METHODS.

The following analytical methods were used :—

Total nitrogen : Kjeldahl method using a Markham still (Markham, 1942) and the catalyst recommended by Chibnall, Rees and Williams (1943) or Kjeldahl-Nesslerisation.

α-Amino nitrogen : the ninhydrin-CO_2 method (Van Slyke, Dillon, MacFadyen and Hamilton, 1941).

Total carbohydrate : anthrone method of Morris (1948) and the orcinol method of Sørensen and Haugaard (1933).

Reducing sugar : Hagedorn and Jensen (1923).

Hexosamine : the method of Elson and Morgan (1933) with the modification of Immers and Vasseur (1950).

Phosphorus : Fiske and SubbaRow (1925).

Amino acids were detected by paper chromatography (Consden, Gordon and Martin, 1944). Solvents used were phenol : H_2O, collidine : lutidine : H_2O and butanol : benzyl alcohol : H_2O.

Sugars were detected by paper chromatography (Partridge, 1949) using collidine : lutidine : H_2O.

The ultraviolet absorption spectra were observed in a Unicam spectrophotometer.

Sedimentation studies were carried out in a Spinco ultracentrifuge.

RESULTS.

Inoculum, strain of *B. anthracis*, media, preparation of lyophilised growth filtrates, alum precipitates of antigen and potency tests are as described in the preceding paper (Belton and Strange, 1954). Lyophilised growth filtrates are referred to as " crude antigens."

The effect of dialysis on the crude antigen.

With synthetic and casein-synthetic media the constituents are all diffusible through a cellophan sac and non-diffusible material remaining would be expected to contain the protective antigen if it were a protein and not denatured by such treatment.

The crude antigen was dissolved in distilled water to give a 10–12 per cent solution. The solution was deeply coloured purple-brown when synthetic or casein-synthetic media were used and this pigment appeared as a result of the growth of the virulent and non-virulent strains of *B. anthracis*, and was perceptible in the medium without concentration. At pH 6·0 it changed to yellow. Spectroscopically it showed no absorption bands and it could not be extracted with common organic solvents. It has not been identified.

The solution was put into cellophan bags and dialysed against frequent changes of distilled water at 2° for 4 days. Some pigment diffused out and a precipitate appeared in the sac : this precipitate was centrifuged off and freeze-dried. The clear supernatant was reduced in volume under reduced pressure and freeze-dried. The yield of soluble non-diffusible material varied with the organism and medium used and was smallest when it was obtained from the " Sterne " strain in synthetic medium (Table I). Potency was tested in the same way as described in the previous paper for crude antigen, that is, one-fifth of the total dose in 0·5 ml. H_2O given in 5 injections at 2-day intervals and challenge with 500 spores of M. 36 strain of *B. anthracis* 7 days later. The results with various preparations are given in Table II and it can be seen that the most active preparation on a weight basis is that obtained from " Sterne " cultures in synthetic medium when 0·2 mg. completely protected each of a batch of 4 rabbits. The precipitate in the sac after dialysis of " Sterne " material was found to be active. In one experiment with synthetic medium, of 4 rabbits each given a total weight of 1 mg., 3 survived challenge. No protective antigen was detected in diffusates from solutions of crude antigens after the growth of the " Sterne " strain.

TABLE I.—*Yield of Non-diffusible Soluble Growth Products per g. of Crude Antigen.*

Medium.	Synthetic.	Synthetic.	Casein-synthetic.	Casein-yeast.
Strain	N.P.A.	" Sterne "	" Sterne "	" Sterne "
Yield (mg.)	15	2–3	3–4	9–10
Appearance	Grey	Pink	Brown	Brown

TABLE II.—*The Protective Activity of Non-diffusible Growth Products.*

Medium	Synthetic.				Synthetic.	Casein-yeast.		
Strain	N.P.A.				" Sterne."	" Sterne."		
Total wt. per rabbit (mg.)	1·0	0·75	0·5	0·25	0·2	1·0	0·5	0·25
No. surviving	4	4	3	2	4	3	4	2
No. in test	4	4	3	4	4	3	4	4

All control animals died.

Components of soluble non-diffusible material.

All samples contained most of the common amino acids, glucosamine and galactose (liberated by acid hydrolysis). N.P.A. material contained a large excess of glutamic acid derived from the capsular polyglutamyl peptide and this explained the higher yield and lower potencies compared with material from non-capsulated organisms. When yeast extract medium was used, acid-hydrolysates of non-diffusible products showed the presence of mannose and glucose coming from the polysaccharides in yeast; these increased yield and reduced potency. The glucosamine and galactose were shown to be present in the form of a polysaccharide similar to the one described by Ivanovics. A proportion of the amino acids was accounted for as a protein with protective activity. Some analytical results for these products are shown in Table III.

TABLE III.—*Composition of Water-soluble Non-diffusible Growth Products.*

Medium and strain.	Ash (per cent).	Total nitrogen (per cent).	α-NH_2 N after hydrolysis (per cent).	Hexosamine after hydrolysis (per cent).	Total C/H as galactose (per cent).	Reducing power after hydrolysis (per cent as glucose).
Synthetic N.P.A.	5·5	9·5	6·6	9·7	12·0	19·4
Synthetic " Sterne "	—	7·0	5·4	12·4	15·0	22·0
Casein-yeast " Sterne "	6·6	7–8	5·3–5·5	4·5–6·0	22–29	26–34

Fractionation of non-diffusible growth products.

Material from N.P.A. was precipitated in aqueous solution with copper sulphate (Bovarnick, 1942) and impure polyglutamyl peptide was obtained. After freeing the precipitate and supernatant from copper by dialysis against citrate buffer at pH 5·0 and freeze-drying, the activity of the 2 fractions was tested, with results as shown in Table IV. Both were active. That the peptide is not the protective antigen is proved by the fact that protective material from the " Sterne " strain contains no peptide. Paper chromatography of acid-

hydrolysed polyglutamyl peptide obtained in these experiments showed mainly glutamic acid but there were traces of other amino acids suggesting a protein impurity.

TABLE IV.—*The Protective Activity of Freeze-dried Copper Precipitates and Soluble Material from N.P.A. Non-diffusible Soluble Material after Freeing from Copper.*

Experiment	1.		2.	
Fraction	Ppt.	Sol.	Ppt.	Sol.
Total wt. given to each rabbit (mg.)	2	2	0·5	0·5
No. surviving	2	4	3	2
No. in test	4	4	4	4

All 4 control animals died.

Precipitation of material in aqueous solution with 45 per cent v/v acetone in the cold was tested but the protection afforded by fractions was poor (Table V).

TABLE V.—*Protective Activity of Freeze-dried* 45 *per cent Acetone Precipitates and Soluble Material.*

Strain	N.P.A.		"Sterne."	
Fraction	Ppt.	Sol.	Ppt.	Sol.
Total wt. given to each rabbit (mg.)	0·5	1·0	0·25	0·28
No. surviving	3	1	3	0
No. in test	4	4	4	3

All 4 control animals died.

Some concentration of active material was obtained by precipitation with 0·1 N acetic acid in the cold, but as Table VI shows, active material remained in solution. Better results were obtained by precipitating material with an equal volume of 0·1 M citric acid : sodium citrate buffer at pH 3·7 in the cold. In one experiment using casein-synthetic medium and the "Sterne" strain, 100 μg. of precipitate protected 4/4 rabbits and 50μg. 3/4.

TABLE VI.—*Protective Activity of* 0·1 N *Acetic Acid Precipitates and Soluble Material.*

Strain	N.P.A.				"Sterne."			
Experiment	1.		2.		1.		2.	
Fraction	Ppt.	Sol.	Ppt.	Sol.	Ppt.	Sol.	Ppt.	Sol.
* Equivalent total wt. of N.D.R. per rabbit (mg.)	1	1	0·7	0·7	0·6	0·6	0·35	0·35
No. surviving	3	3	4	0	4	2	4	1
No. in test	3	3	4	4	4	4	4	4

* The precipitate and soluble fractions were not weighed but given to animals on the basis of weight of non-diffusible residue (N.D.R.) precipitated with acetic acid. All control animals died.

The most satisfactory results were obtained with trichloracetic acid (TCA) in the cold. Using N.P.A. material, 0·35 mg. of a neutralised, dialysed TCA precipitate protected all of 4 rabbits, whereas 0·4 mg. of the soluble fraction had no protective activity. The active material contained a large proportion of polyglutamyl peptide and it was found that when this was absent, as with similar precipitates from " Sterne " material, very small amounts would protect. For subsequent work this latter type of material was obtained by direct precipitation of concentrated growth medium with TCA.

The Isolation of Antigen and Polysaccharide from Casein-synthetic Medium after Growth of the " Sterne " Strain of B. anthracis.

All operations were carried out at 0° using a water-glycol bath and a M.S.E. refrigerated centrifuge. Reagents were cooled before use. Batches of 20 l. of glass-filtered medium were lyophilised in a stainless steel drum, giving approximately 150 g. of powder. This was dissolved in 900–1000 ml. of distilled water and cooled to 0° when a crystalline deposit was obtained which was centrifuged down and rejected. On the addition of 50–60 ml. 6 N-HCl with vigorous stirring, to make the pH slightly acid, the solution turned yellow. TCA (25 per cent) was then added slowly to give a concentration of 2·5 per cent and the solution left at 0° for ½ hr. The precipitate was centrifuged down, washed twice with 2·5 per cent TCA and dissolved in 15–20 ml. 0·5 M-$NaHCO_3$, any insoluble material being centrifuged down and rejected. The clear solution was freed from salts by dialysis in the cold, when material tended to come out of solution. At one stage the precipitate so formed was separated from the soluble fraction but when it was found that it had activity, the contents of the sac were freeze-dried without separation. Yields of this material (fraction A) varied but were approximately 0·7 mg. per g. of original powder.

The TCA supernatant was neutralised with solid $NaHCO_3$ (a little capryl alcohol being added to prevent foaming) and dialysed against frequently changed distilled water in the cold. After dialysis the insoluble portion was centrifuged down and freeze-dried giving fraction B.

The clear supernatant was reduced in volume under vacuum to 30–50 ml. when more material came out of solution, which was separated, freeze-dried and added to *B. Ethanol* was then added to 25 per cent concentration with a few drops of saturated potassium acetate and after standing ½ hr., the small precipitate which formed was centrifuged down; the supernatant was separated and treated with alcohol to make a total of 3 vol. and allowed to stand for ½–1 hr. The precipitate was centrifuged down, dissolved in water, re-precipitated with 3 vol. alcohol, the centrifuged precipitate dissolved in water, treated with active charcoal, filtered through a 5/3 sintered glass filter (after centrifuging down the charcoal), dialysed in the cold and freeze-dried. Yields of this material (fraction P) were about 1 mg./g. original powder. The alcohol supernatant after freeing from alcohol and dialysis was found to contain a small amount of hygroscopic non-diffusible material.

This scheme is shown in Fig. 1.

Activity of Fractions.

As small quantities of material were involved, the 5 doses for a batch of rabbits were usually prepared together and kept at −15° until required. If the

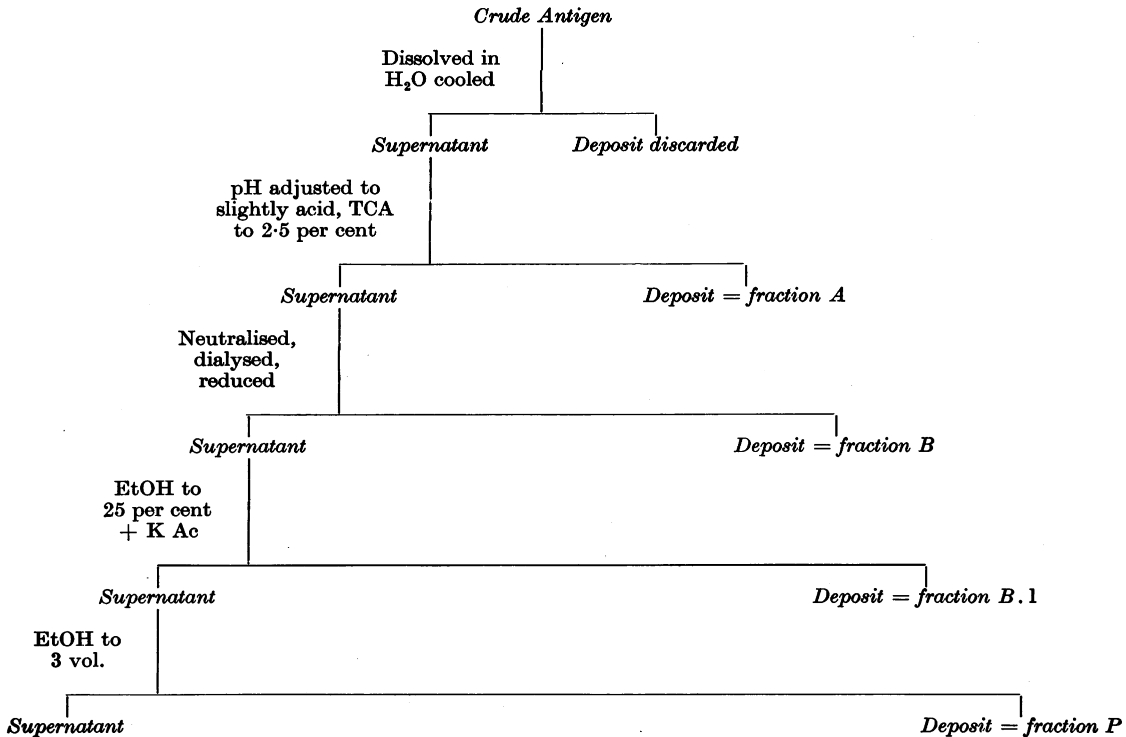

FIG. 1.—Scheme for the fractionation of crude antigen.

material was not completely soluble in water it was dissolved in weak bicarbonate solution. Results of 4 preparations of fraction A are given in Table VII and it may be seen that using F. 99, 5 injections each of 5 μg. protected a batch of 4 rabbits. The potency of fraction B varied; in one experiment 200 μg. per rabbit protected 3 out of 4.

TABLE VII.—*Activity of Trichloracetic Acid Precipitates from Casein-Synthetic Medium (Fraction A).*

Sample.	F. 34.		F. 82.			F. 99.		F. 123.
Total wt. per rabbit (μg.)	75	45	100	45	20	50	25	50
No. rabbits surviving	4	4	4	3	2	4	4	3
No. in test	4	4	4	4	3	4	4	3

All 4 control rabbits died.

Analysis of Fractions.

Fraction A.—A sample was taken from the pooled material of 3 batches and re-precipitated 3 times with TCA, the precipitate being dissolved in 0·1 M-$NaHCO_3$ each time. After the last precipitation it was dialysed in the cold

and lyophilised. The resulting material, when dry, was light brown but was more strongly coloured in solution. Qualitative tests on a 0·5 per cent solution gave the following results :—

Molisch : weak positive ; biuret : positive (rose-violet) ; Millons (Cole's modification) : positive ; Hopkins-Cole : positive ; xantho proteic : positive ; Sakaguchi : strong purple colour.

Quantitative analysis gave the following results :—ash : 2·4 per cent ; total nitrogen : 14·2 per cent. ; α-amino nitrogen after hydrolysis with 6 N-HCl for 20 hr. : 10·7 per cent. ; phosphorus : 0·07 per cent. ; total carbohydrate : < 1 per cent (using the anthrone method a brownish-green colour was obtained which gave an optical density equivalent to 0·8 per cent galactose) ; hexosamine : 0·5 per cent. Paper chromatography after acid hydrolysis of the material with 6 N-HCl for 20 hr. showed the presence of the following amino acids : cystine, glutamic, serine, glycine, alanine, threonine, lysine, arginine, histidine, valine, leucine, isoleucine, tyrosine, proline, phenylalanine and methionine.

Ultraviolet absorption : a solution containing 0·6 mg./ml. in 0·05 M-$NaHCO_3$ after centrifuging in an angle centrifuge at 18,000 r.p.m. for 20 min. showed a general absorption over the range 2300–3200 Å typical of that expected from a protein material, but the trough around 2500 Å was shallow.

Ultracentrifuge : 2 runs were made by Dr. B. Record of this department using 1 per cent solutions in glycine : sodium hydroxide buffers at pH 8·5 and 11·0. The sedimentation boundary broadened rapidly as the run proceeded and did not draw clear of the meniscus. The material sediments as a single boundary with a sedimentation constant equal to 3·0. Whether the boundary spreading is due to some polydispersity in the material or can be accounted for by normal diffusion cannot be decided from this brief investigation. It is clear, however (see Fig. 2), that the material sediments as a single boundary with a sedimentation constant of 3·0 Svedbergs.

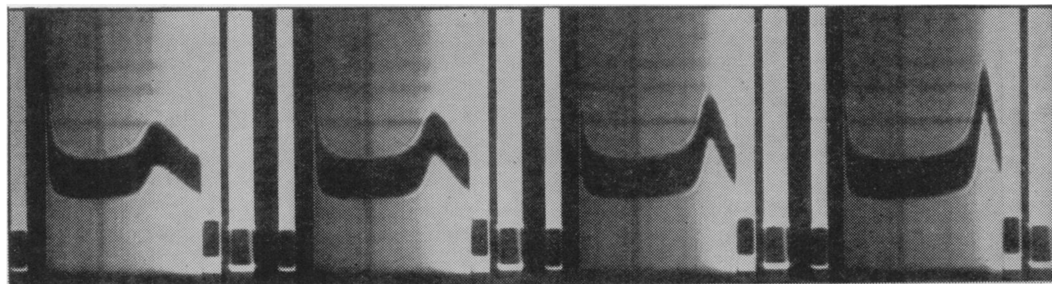

FIG. 2.—Sedimentation pattern of fraction A, 1 per cent w/v, speed 59,780 r.p.m. Sedimentation from right to left in glycine : sodium hydroxide : sodium chloride buffer, pH 11·0, I = 0·2. Four exposures at 32, 48, 64 and 80 min. intervals after reaching full speed.

It can be seen from the sedimentation diagram that the pigment accompanies the sedimenting boundary throughout the run suggesting that the pigment is combined with, or part of, the main bulk of sedimenting material.

Fraction B.—Complete analysis has not been made because the material was obviously impure. It was very strongly pigmented. Qualitative tests on a 0·5 per cent solution in 0·05 M-$NaHCO_3$; Molisch : positive ; biuret : positive

(red violet) ; xanthoproteic : positive ; Millons : weak positive ; Hopkins-Cole : positive.

Ash : 8·6 per cent ; total nitrogen : 8·1 per cent ; total carbohydrate (as galactose) : 4 per cent (anthrone). The ash contained 21·0 per cent Fe.

Fraction P.—The freeze-dried material was white and dissolved readily in water giving a slightly brown solution.

Qualitative tests on a 1 per cent aqueous solution ; Molisch : strong positive ; biuret : negative ; xanthoproteic : negative.

Total nitrogen : 3·9 per cent ; α-amino nitrogen : 1·26 per cent ; ash : 0·7 per cent.

Total carbohydrate : 43 per cent (anthrone), 39·4 per cent (orcinol).

Reducing power : the total reducing power was obtained after hydrolysis with 0·5 N-HCl for 5 hr. : 66·7 per cent as glucose.

Hexosamine : all the hexosamine was liberated after hydrolysis with 3 N-HCl for 3 hr. : 34·0 per cent (as base).

Acetyl (hydrolysed with *p*-toluene sulphonic acid followed by steam distillation of volatile acid) : 14·3 per cent.

Sugars detected by paper chromatography after 0·5 N-HCl hydrolysis : galactose only.

Amino acids (paper chromatography) ; alanine, aspartic and glutamic with traces of others. Hexosamine showed up strongly with the spray.

Sedimentation studies in the Spinco ultracentrifuge of the material in acetate : sodium acetate buffer at pH 4·0 and phosphate buffer at pH 8·0 using a 1 per cent solution showed only one component (Fig. 3).

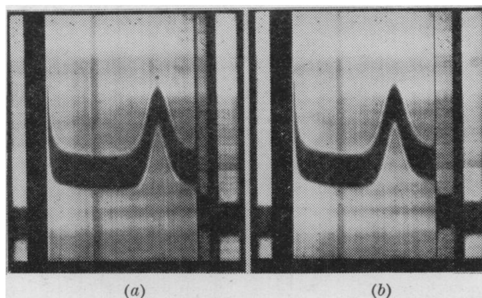

FIG. 3.—Sedimentation patterns of fraction P, 1 per cent w/v, speed 59,780 r.p.m. Migration from right to left. (*a*) In acetate : sodium chloride buffer, pH 4·5, I = 0·2. (*b*) In phosphate : sodium chloride buffer, pH 8·0, I = 0·2. Exposure after 128 min. at full speed in each case.

The polysaccharide was examined by Professor W. T. J. Morgan at the Lister Institute who detected no blood group A, B or H activity.

Other fractions.

The 25 per cent alcohol-precipitated material from crude antigen was mainly polysaccharide and showed small variable activity. The 3 vol. EtOH-soluble material was mainly peptide.

From the analytical results it is seen that fraction A has the characteristics of a protein, fraction B consists of some protein with inorganic material and

pigment and fraction P is a polysaccharide similar to that described by Ivanovics (1940).

The isolation of protective antigen from alum precipitates of growth filtrates.

The use of citric acid as a solvent for such precipitates was suggested by Dr. D. Herbert of this department. From citric acid solutions of alum precipitates, active material was obtained either by adjusting the pH to 3·5 with sodium citrate or by adding TCA. Another method tried was to stir the material in 2·5 per cent TCA when the alum dissolved and antigen was left as a precipitate.

The citric acid-TCA method was as follows : the alum precipitate equivalent to 20 l. of growth filtrate was packed down by centrifugalisation and washed with saline. It was dissolved at 2° in 1 l. of 0·2 M citric acid by stirring, the process requiring about 1 hr. : 1/10th vol. of cold 25 per cent TCA was added slowly with stirring and the mixture left at 0° for $\frac{1}{2}$ hr. The faint precipitate was centrifuged down and washed twice with cold 2·5 per cent TCA. The residue was extracted 4 times with 5 ml. 0·2 M-$NaHCO_3$ by centrifugalisation, these extracts being decidedly pink when the medium used was casein-carbon. The extracts were mixed, dialysed in the cold and lyophilised yielding 40–45 mg. of brownish-grey solid with casein-carbon medium and 52 mg. with casein-yeast medium. The $NaHCO_3$-insoluble residue consisted of white crystalline material with a small gelatinous layer, probably denatured protein.

Activity of antigen from alum precipitates.

Table VIII shows the protection given to batches of rabbits by these preparations. Samples F. 73, F. 76 and F. 79 were isolated from casein-yeast medium and F. 155 from casein-carbon medium. F. 155 had somewhat higher activity than the others and was as potent as fraction A.

TABLE VIII.—*Activity of Material Isolated from Alum Precipitates of Growth Filtrates.*

Sample	F. 73.			F. 76.			F. 79.			F. 155.		
Total wt. per rabbit (µg.)	100	70	35	100	70	35	100	70	20	100	50	25
No. rabbits surviving	4	3	2	4	2	1	3	1	2	3	3	4
No. in test	4	3	4	4	4	4	3	3	4	3	4	4

All 4 control rabbits died.

Analysis of antigen from alum precipitates.

These preparations resembled fraction A in giving protein reactions and containing little carbohydrate or hexosamine. Material derived from casein-carbon medium dissolved in weak $NaHCO_3$ to give a pinkish solution. Total nitrogen varied between 12–13 per cent but after re-precipitation of a batch 3 times with $\frac{1}{2}$ saturated $(NH_4)_2SO_4$ and thorough dialysis this increased to 14·7 per cent. The ultraviolet absorption spectrum of the re-precipitated material was similar to that of fraction A.

Attempts to increase yield of antigen from alum precipitates.

The yield of antigen obtained from alum precipitates was somewhat lower than expected and the TCA supernatant was examined in an attempt to increase it. The supernatant from a batch was neutralised and dialysed against water in the cold. On lyophilisation a large amount of material was obtained which analysis showed to be mainly inorganic (ash, 54 per cent) suggesting the presence of a complex aluminium compound. Of 4 rabbits receiving 100 mg. each of this material, only one survived challenge which is an insignificant activity. In another experiment the supernatant from a batch was neutralised with solid $NaHCO_3$ and after dialysis the volume was reduced under vacuum to about 40 ml. Treatment with TCA in the cold gave an insignificant amount of precipitate.

Aggressin activity of protective antigen fractions.

Keppie, Smith and Harris-Smith (1953) have pointed out an interesting correlation between aggressin activity as evidenced by interference with phagocytosis, and protective antigen activity in fractions obtained from guinea-pigs infected with *B. anthracis*. Antigen preparations described above have been tested by them and found active at 0·1 per cent. This is additional support for correlation of the two activities.

Further purification of protective antigen fractions.

An apparent purification was effected by extracting the preparations described with 0·2 M-Na_2HPO_4 : 0·1 M citric acid buffer pH 5·0 when some material went into solution (less than 20 per cent). After dialysing and lyophilising the 2 fractions they appeared different, the soluble being white and the insoluble brown. The ultraviolet absorption spectrum of the soluble fraction was more typical of a pure protein than the insoluble fraction (Fig. 4). However, animal experiments showed that protective activity had not been concentrated in either fraction but was approximately the same for each and apparently lower than that of the original preparation.

Re-fractionation of the preparations with acetone or ammonium sulphate has not resulted in fractions with high protective activity.

Increase in protective activity of antigen preparations after treatment with horse-serum.

F. 123 (1 mg.) was incubated at 37° for $\frac{1}{2}$ hr. with 0·2 ml. of normal horse serum and left at 2° for 48 hr. The mixture was diluted with saline and 5 doses containing 2 μg. of antigen and approximately 32 μg. of horse-serum proteins were given to each of 4 rabbits. Three of these survived challenge whereas an equal amount of antigen without this treatment failed to protect any of a batch of 4 rabbits. Similarly 5 doses of 2 μg. of F. 155 and 50 μg. of protein protected 2 of 3 rabbits whereas in the absence of serum this amount would not protect.

DISCUSSION.

The evidence presented here suggests that the protective antigen of *B. anthracis* is a protein or closely associated with one. It may be concentrated in crude

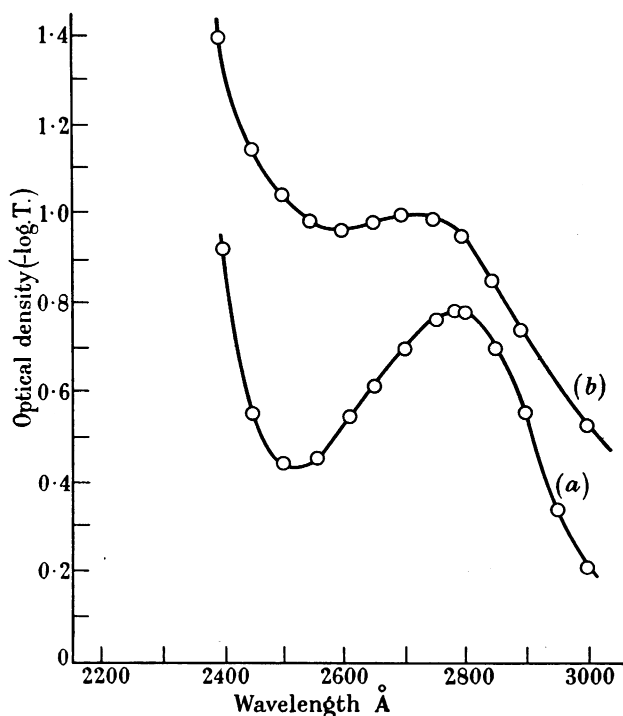

FIG. 4.—Ultraviolet absorption of the 2 fractions obtained from fraction A by phosphate : citrate buffer extraction at pH 5·0. Concentration 0·06 per cent in 0·05 M-$NaHCO_3$ in each case (*a*) soluble ; (*b*) insoluble.

antigen by dialysis and lyophilisation but after growth of the " Sterne " strain the activity recovered appears to be lower than that originally present in the growth filtrate whereas after growth of the capsulated N.P.A. strain practically all the activity is recovered. It is possible that free capsular polyglutamyl peptide acts as a preservative for protective antigen during dialysis and it is perhaps significant that this peptide, when first isolated, is contaminated with it. Investigation of dialysates showed that a method of further concentrating antigen was treatment with TCA in the cold. While it is well known that the effect on proteins of this reagent can be drastic, the results obtained seemed to justify its use since milder reagents did not give a greater yield with reference to activity recovered in precipitates.

The isolation of similar highly active fractions as TCA precipitates from concentrated growth filtrates and citric acid solutions of alum precipitates is significant since alum precipitation itself effects a considerable purification of antigen. However, it is possible that our preparation is not homogeneous since the method of isolation merely concentrates protein. The ultracentrifuge analysis of fraction A was made in an attempt to discover whether more than one component was present, and while the results were by no means satisfactory, the sedimentation diagram obtained could be that of a homogeneous protein. Although apparent separation was achieved by citrate : phosphate buffer extraction

at pH 5·0, both fractions were active and neither more active than the original preparation so that insolubility in slightly acid solution of a large proportion of the material could be due to partial denaturation during the process of isolation.

The increase in protective activity of antigen preparations after treatment with horse serum is interesting since it could explain the apparent higher yields obtained by Gladstone (1946; 1948) using medium containing serum compared to the yields obtained in non-protein medium.

Further work is required to show whether these antigen preparations are relatively pure or contain a non-specific bacterial protein.

The polysaccharide was investigated in order to compare its chemical constitution with that of the polysaccharide described by Ivanovics (1938) and our analytical results suggest that it is very similar although our ratio of hexosamine to galactose is not 1 : 1. In agreement with him we have been unable to obtain this polysaccharide free from amino acids.

SUMMARY.

After *B. anthracis* has grown in a non-protein medium it is possible to isolate from the culture filtrate a mixture of growth products which contains protective antigen.

The components of such growth products vary according to the strain of organism and the type of medium used but a protein and a galactose : glucosamine polysaccharide are always present.

The protein has been isolated from growth filtrates and has the property of a strong protective antigen against anthrax, 25 μg. protecting rabbits against a challenge dose of 250 A.L.D. of spores. Although no definite criteria of purity are presented the sedimentation patterns obtained could be those of a homogeneous substance.

A polysaccharide has been isolated from growth filtrates which sedimented as a homogeneous substance and was similar to that described by Ivanovics.

It is a pleasure to record our thanks to Dr. D. W. Henderson and Dr. D. Herbert of this department for advice and interest. We are indebted to Mr. F. A. Dark for some analytical and fractionation work, Dr. B. Record and Mr. K. Grinstead for sedimentation studies and freeze-drying and Mr. C. F. B. Boardman for technical assistance.

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