

STUDIES ON A PROTECTIVE ANTIGEN PRODUCED *IN VITRO* FROM *BACILLUS ANTHRACIS*: MEDIUM AND METHODS OF PRODUCTION.

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

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

Received for publication December 21, 1953.

It has long been known that an extracellular protective antigen is produced when *Bacillus anthracis* is grown *in vivo*. Bail (1904) demonstrated the immunising action of filtrates of oedema fluid from infected animals, and this was confirmed by Staub and Grabar (1944) and Cromartie, Watson, Bloom and Heckly (1947). Both tried to purify the antigen, but the task proved difficult because of other proteins and polysaccharides in oedema fluid; the preparations obtained were not homogeneous.

The nutritional factors and growth conditions necessary for the elaboration of protective antigen *in vitro* are not well understood, but they are certainly distinct from the factors necessary for growth, since in media in which excellent growth occurs antigen is frequently not produced.

The first successful production of protective antigen *in vitro* was reported by Gladstone (1946) who obtained it by growing the organism under defined conditions in media containing plasma or serum. He showed that it was a thermolabile, extracellular substance, present in culture filtrates and not in the bacterial cells. The factors in serum necessary for antigen production appeared to be bicarbonate and the albumin fraction, and it was later found possible to produce antigen in broth containing bicarbonate and purified bovine serum albumin (Gladstone, 1948). These media provide the most potent *in vitro* sources of antigen, but the actual quantity of antigen in the filtrates is small compared with the amount of serum proteins, and isolation of the pure antigen is difficult. For work on the chemical nature of the antigen, media free from high molecular weight materials is to be preferred.

More recently Wright, Hedberg and Slein (1954) obtained consistent, if relatively small, yields of protective antigen in a synthetic medium without protein or other macro-molecules. This medium was based on the synthetic medium of Brewer, McCullough, Mills, Roessler, Herbst and Howe (1946) and contained 17 amino acids, adenine, guanine, uracil, thiamine, KH_2PO_4 , K_2HPO_4 , CaCl_2 , MgSO_4 , MnSO_4 , FeSO_4 , Na HCO_3 , glucose and glutamine. After growth of a non-proteolytic virulent strain of *B. anthracis* for 20 hr. the culture was filtered through sintered glass, and the filtrate lyophilised. The resulting powder was re-constituted in water and used as a protective vaccine given in 5 subcutaneous doses. The antigen could be precipitated with alum, and only 2 doses of this were required.

The work described in this paper was undertaken to find the conditions necessary to increase the amount of antigen per ml. of filtrate, and a method for

large scale production so that purification and analytical studies could be carried out; it was commenced on the basis of Wright's medium. As it seemed preferable to use a protein hydrolysate rather than synthetic amino acids, the first modification was the use of an acid casein hydrolysate with certain amino acids. It was found that the method of preparation of the hydrolysate was important since HCl hydrolysis of commercial casein followed by neutralisation and decolourisation with charcoal did not provide a preparation suitable for antigen production, even with amino acid additions. A satisfactory product was obtained by hydrolysing casein after alcohol extraction and successive solution in NaOH, followed by acid precipitation as described by Barton-Wright (1944). The hydrolysate was lyophilised, its α $\text{NH}_2\text{-N}$ content found and a weight equivalent to 70 mg. α $\text{NH}_2\text{-N}$ was added to every 250 ml. of final medium. It was necessary to add tryptophan, and better antigen yields were obtained if glycine and cystine were also added. Excellent results were obtained using commercial preparations such as Ashe vitamin-free casein hydrolysate or Difco casamino acids, and the latter has been adopted by us since the former is no longer obtainable.

The second modification was the use of yeast extract to supply nucleic acid components and thiamine. This was prepared as described by Gladstone and Fildes (1940) and contained approximately 0.1 per cent total nitrogen, 1.3 per cent total solids which gave 40-50 per cent ash. About 80 per cent of the solids were dialysable through a cellophan sac and the non-dialysable material contained yeast nucleic acid and a mannan among other things. The dialysable material allowed the production of some antigen but the whole yeast extract was necessary for good yields. To every 250 ml. of final medium 3 ml. of a typical yeast extract was added. The other constituents were as defined by Brewer and used in the concentrations described by Wright.

A third modification was the addition of charcoal, since when a highly activated wood charcoal was included in the medium in optimum quantities, the potency of the final filtrate was increased. The amount of growth and the final pH were unaffected. The charcoal appears to adsorb and remove some inhibitory substance which is produced by the organisms during growth. When the charcoal was added to the basal medium, shaken up frequently for 1 hr. at 37° and then removed by filtration before or after autoclaving, the potency of the filtrate after growth was equivalent to that from non-charcoal medium, but if sterile charcoal was added aseptically to the autoclaved basal medium and left there during incubation the increase in potency was obtained. For convenience it was usual to add it to the basal medium before autoclaving.

EXPERIMENTAL.

Inoculum.

Two strains of *B. anthracis* were used, the virulent non-proteolytic strain N.P.A., a mutant from "Vollum" isolated after exposure to ultraviolet light (Wright, Hedberg and Feinberg, 1951) and the avirulent "Weybridge." This strain was isolated by Sterne (1937) and used by the Ministry of Agriculture and Fisheries as a living spore vaccine.

The inoculum consisted of a washed and heated spore suspension, 1×10^8 spores being added to 500 ml. of medium.

The parent strains were dried in small tubes (Stamp, 1947) and stored over phosphorus pentoxide. A fresh stock of concentrated spore suspension was prepared every 3 to 4 months by suspending the contents of a tube of dried spores in 1 ml. of warm tryptic digest broth and subculturing into broth, transferring to agar slopes and incubating overnight at 37°.

The cultures were washed off with 5 ml. sterile distilled water per slope and used to inoculate C.C.Y. (Gladstone and Fildes, 1940) agar in 8 oz. medical flats. These were incubated for 2 days at 37° to obtain maximum sporulation. The spores were washed off with 25 ml. distilled water per medical flat, pooled, washed 4 times in ice-cold distilled water, re-suspended to approximately one-tenth the original volume, heated to 60° for 90 min. and the viable count estimated. The count was usually between 1×10^{10} and 5×10^{10} spores per ml. The concentrated spores were stored at 2°. The viability was checked frequently and remained constant. The stock spore suspension was diluted as required for the inoculation of medium.

Virulence checks were carried out on every batch of concentrated "Weybridge" spores produced. Mice in groups of 10 were given spores intraperitoneally in 0.1 ml. ranging from 10^3 – 10^6 per mouse in 10-fold concentrations. The mice were kept for 14 days. No anthrax deaths occurred during this time in the test mice but all controls given 10^3 spores of a virulent strain were dead by the fourth day.

Medium.

The water used in making the medium was distilled and then passed through a column of Amberlite Monobed resin M.B. 1 (analytical grade) obtained from British Drug Houses Ltd. Inorganic salts and glucose were "Analar" grade; amino acids and growth factors were British Drug Houses laboratory reagents.

Six stock solutions, A, BI, BII, C, D and E were prepared. A, BI, BII and C may be kept for long periods in the cold but D and E are not stable and were prepared within twenty-four hours of use.

Solution A contained Difco casamino acids, 90 g.; glycine, 0.375 g.; L-tryptophan, 1.3 g.; L-cystine, 0.3 g.

The tryptophan was dissolved separately by stirring in 10 ml. N-HCl and adding more acid in 1 ml. volumes as required. The cystine was mixed with 3 ml. H₂O and concentrated HCl added drop by drop with stirring until it had dissolved. These two solutions were added to the casein and glycine dissolved in water and the volume made up to 1 l. The pH was approximately 5.1.

Solution BI, for use in casein-synthetic medium, contained CaCl₂ 6H₂O, 0.5512 g.; MgSO₄ 7H₂O, 0.25 g.; adenine, 0.07 g.; guanine, 0.075 g.; uracil, 0.07 g.; thiamine, 0.0025 g. The guanine was made into a paste with 20 drops of concentrated HCl and water added until it dissolved. The other solids were dissolved separately in water and mixed with it in the order given. The resulting solution was diluted to 1 l. The pH was 3.2 to 3.3.

Solution BII, for use in casein-yeast medium, contained CaCl₂ 6H₂O, 0.5512 g.; MgSO₄ 7H₂O, 0.25 g.; yeast extract, 300 ml.; made up to 1 l. Although the required volume of yeast extract is not critical it will need to be adjusted if the total nitrogen is considerably more or less than 0.1 per cent.

Solution C contained KH₂PO₄, 34.0 g.; KOH, 5 per cent (w/v), 160.0 ml. The phosphate was dissolved in approximately 800 ml. water and the hydroxide solution added until the pH was 6.8. The volume was made up to 1 l.

Solution D contained NaHCO₃, 25 g.; D-glucose, 10 g.; glutamine, 0.01 g. The solids were dissolved in water and the volume made up to 900 ml. The solution was sterilised by filtration.

Solution E contained FeSO₄ 7H₂O, 2.78 g.; MnSO₄ 4H₂O, 0.0223 g. The ferrous sulphate was dissolved in 200 ml. water containing 0.3 ml. concentrated HCl, mixed with the manganese sulphate dissolved in water and the volume made up to 400 ml. The solution was sterilised by filtration. Finally 0.1 ml. of a concentrated spore suspension was added aseptically so that 10 ml. contained 1×10^6 spores.

Charcoal.

The charcoal used was a fine highly activated wood type conforming to the following specifications: Code No. N. 182, retained on 325 mesh screen, 1.9 per cent; maximum particle diameter, 65 μ ; diameter of bulk of particles, 20–40 μ ; specific gravity, 0.331.

The optimum quantity required was found to be within reasonably fine limits and when excess was used all of the antigen was lost due to the adsorption of both it and inhibitor. The amount required of the batch used was 0.2 to 0.1 per cent (w/v). Decreasing amounts

down to 0.02 per cent were still partially effective but no protection was obtained with cultures containing 1 per cent.

Undoubtedly the optimum quantity will vary with each batch of charcoal, depending on its adsorptive power. The amount required of another batch of similar charcoal was approximately one-half to one-quarter of the above figures.

Basal medium.

This was prepared in Thompson bottles (approximately $27 \times 15 \times 8$ cm., obtained from United Glass Bottle Co. Ltd.) by adding 20 ml. of solutions A, BI or BII and C to 385 ml. water in each bottle. For charcoal-casein medium the optimum amount of charcoal was added and the bottles plugged with cotton wool. They were sterilised by heating in flowing steam for 1 hr. and autoclaving for 20 min. at 5 lb./sq. in. followed by 20 min. at 15 lb./sq. in. The pH of the basal medium after sterilisation was 6.9. The bottles were stored at 2° until required.

Final medium.

To complete the medium, 45 ml. of solution D and 10 ml. of solution E (containing spore inoculum) were added aseptically to each Thompson bottle giving a final volume of 500 ml. The addition of E produced a colloidal opalescence which was desirable for antigen production: the pH of the medium was 7.4 to 7.5.

Cultures.

The Thompson bottles were incubated lying flat for 22 hr. at 37°, affording a layer of medium 17–20 mm. in depth. The optimum incubation time was between 18 and 36 hr.; with longer incubation the potency fell. A convenient time for production of the antigen was approximately 22 hr. and this was adopted as standard.

The pH of the cultures after incubation was 7.3 to 7.4. The opacity of cultures grown in the synthetic medium or the casein-synthetic medium was 3 to 4 by Brown's opacity tubes. The opacity of cultures grown in charcoal-casein medium could not be determined owing to interference by charcoal.

After incubation the cultures were pooled, centrifuged and filtered in the cold.

Filtration.

Various types of filter were tried for sterilising the culture supernatant. Seitz asbestos pads and Berkefeld filters were shown to adsorb and retain the antigen. For experimental work sintered glass bacteriological grade filters, average porosity 1.6μ , were used, but for large scale production millipore filter discs (Lovell Chemical Co., Mass., U.S.A.) fitted into a modified compound glass Seitz filter were used.

The supernatant from cultures inoculated with the virulent N.P.A. strain filtered slowly owing to the presence of polyglutamic acid from capsular material which increased the viscosity of the fluid. The avirulent "Weybridge" strain, being devoid of capsules, filtered considerably faster and required much less negative pressure.

Concentration of the antigen.

Two methods were used for concentration and storage of the antigen, lyophilisation and alum precipitation.

Lyophilisation.—The sterile filtrate was freeze-dried in 3 lb. (1.35 kg.) Kilner jars each containing 200 ml. of filtrate. The weight of the resultant dry powder was approximately 1 g. from 200 ml. of synthetic medium and 1.3 g. from the casein-synthetic or charcoal-casein media filtrates. The material was very hygroscopic and was stored in a desiccator over phosphorus pentoxide.

Alum precipitation.—The antigen was precipitated from the filtrate by the addition of 0.1 per cent aluminium potassium sulphate (Wright *et al.*, 1954). The pH was adjusted to 5.9 with *N*-HCl and the mixture left to settle overnight at 2°. The alum settled to approximately one-tenth of the original volume and the clear supernatant was aseptically removed. After standing for a further few days at 2° the alum sedimented to less than half the volume. All the clear supernatant was again removed and the volume of alum adjusted with distilled

water to one-twentieth that of the original filtrate. Merthiolate 1/5000 was added as a preservative. Stored at 2°.

Preparation of vaccines.

In the absence of an *in vitro* test for estimating potency an active immunisation test was used. Rabbits of heterogeneous strains weighing between 4.5 and 5.5 lb. (2-2.5 kg.) each were used in groups of 4 or 10. Rhesus monkeys weighing between 8 and 12 lb. (3.6-5.4 kg.) each were used in groups of 10.

Two methods of immunisation were used.

(1) The lyophilised powder was reconstituted in distilled water to a concentration equivalent to that of the original filtrate. The weight of the freeze-dried powder from the synthetic medium was 5 mg. per ml. and that from the casein-synthetic 6.5 mg. per ml. Five doses of 0.5 ml. each at 2-day intervals were given subcutaneously. The animals were challenged 7 days after the last dose of vaccine.

(2) Alum-precipitated antigen was diluted in physiological saline to a concentration equivalent to that of the original filtrate or less. Two injections of 1.25 ml. were given at 10-day intervals. The animals were challenged 7 days after the last injection.

To avoid interference from the possible development of local immunity, all vaccines were given subcutaneously into the left flank of the animal and the challenge dose of spores intradermally into the right flank.

Challenge dose.

The average lethal dose for rabbits of the M. 36 strain of *B. anthracis* used for challenge is 2 spores or less. The challenge dose was 500 spores or approximately 250 A.L.D.; this invariably killed all controls by the fourth day. Immunised animals were observed for 14 days.

Monkeys were challenged by the inhalation route. The L.Nt. 50 (Druett, Henderson, Packman and Peacock, 1953) of the M. 36 strain is approximately 4×10^4 spores/litre cloud. The estimated number of spores inhaled by each monkey was between 4×10^5 and 6×10^5 . Controls given no vaccine were usually dead by the 6th day; surviving test animals were retained for 12 months.

RESULTS.

Potency of the antigen.

The animal protection obtained after the appropriate course of vaccines, prepared from the various media filtrates and followed by the standard challenge, are detailed in Table I, II and III.

TABLE I.—*The Potency of Re-constituted Lyophilised Filtrates of Various Media after the Growth of the N.P.A. and "Weybridge" Strains of B. anthracis when Tested in Rabbits.*

Medium. Strain.	Synthetic.				Casein-synthetic.				Casein-yeast.			
	N.P.A.		"Weybridge."		"Weybridge."				"Weybridge."			
Total volume of filtrate per rabbit (ml.)	12.5	2.5	12.5	2.5	12.5	2.5	1.25	0.5		2.5	1.25	0.5
Rabbits surviving	19	6	107	78	21	79	30	16		32	11	16
Rabbits in test	21	13	112	126	22	79	31	41		32	15	32

Vaccines given in 5 doses at 2-day intervals. All control animals died.

TABLE II.—*The Potency of Alum Precipitates from Filtrates of Various Media after the Growth of the "Weybridge" Strain of B. anthracis when Tested in Rabbits.*

Medium.	Synthetic.			Casein-synthetic.			Casein-yeast.			Charcoal-casein.		
	12.5	2.5	0.5	2.5	1.25	0.5	2.5	1.25	0.5	0.5	0.17	0.1
Total volume of filtrate per rabbit (ml.)	. 12.5	2.5	0.5	. 2.5	1.25	0.5	. 2.5	1.25	0.5	. 0.5	0.17	0.1
Rabbits surviving	. 13	8	0	. 10	10	2	. 19	19	12	. 86	52	5
Rabbits in test	. 13	12	12	. 10	10	5	. 19	19	20	. 92	86	20

Vaccines given in 2 doses at 10-day intervals. All control animals died.

TABLE III.—*The Potency of Reconstituted Lyophilised Growth Filtrate and Alum Precipitates of Growth Filtrates after the Growth of "Weybridge" Strain when Tested in Monkeys.*

Medium. Type of vaccine.	Synthetic. Lyophilised powder.		Casein-yeast. Alum ppt.		
	12.5	2.5	2.5	1.25	0.5
Total volume of filtrate per monkey (ml.)	. .	12.5 2.5	. 2.5	1.25	0.5
Monkeys surviving	. .	10 7	. 9	7	6
Monkeys in test	. .	10 10	. 9	9	10

All control animals died.

Toxicity tests.

Two forms of toxicity tests have been carried out in guinea-pigs and rabbits with alum-precipitated antigen.

Intradermal test.—The thorax and abdomen of three guinea-pigs and three albino rabbits were shaved. At six selected points 0.25 ml. of the following were given intradermally:—alum-precipitated antigen 20-fold concentration; alum-precipitated antigen 5-fold concentration; alum-precipitated antigen equivalent to filtrate; alum-precipitated antigen 1/5 of the equivalent of filtrate; alum-precipitated antigen 1/25 of the equivalent of filtrate, and as a control 0.25 ml. of physiological saline.

These animals were observed daily for 4 weeks; at no time was any erythema or other reaction observed. The "button" of alum remained in the tissue throughout.

General toxicity test.—A solution (5 ml.) containing 5 times the concentration of alum-precipitated antigen contained in the original test material was given subcutaneously into the left flank of each of 6 guinea-pigs and 6 rabbits. Observations were made daily for 4 weeks. These animals remained healthy.

Large scale production of the antigen.

To facilitate the study of the chemical nature of the antigen, the production rate was increased to 1000 l. of culture medium per week. Cultures were grown

in Thompson bottles in charcoal-casein medium for 22 hr. at 37°, and after incubation, were syphoned into 20 l. glass containers and clarified by passing through a Sharples centrifuge at 30,000 r.p.m. The supernatant was collected in large stainless steel tanks.

Using a positive pressure of approximately 4 lb. per sq. in. the supernatant was filtered through a modified compound glass Seitz filter containing ten 14 cm. millipore filter discs. The filtration system was completely closed and 230 l. were filtered in 5 hr. The filtrate contained in twenty-three 10 l. aspirators was precipitated with alum (0.1 per cent final concentration of alum) at pH 5.9 and stored at 2° as a 20-fold concentrate.

Aliquots of alum-precipitated antigen taken from samples of filtrate collected at the beginning, middle and end of the filtration process were pooled and tested for antigenicity. The results are shown in Table IV.

TABLE IV.—*The Potency of Alum Precipitate of Filtrate after the Growth of the "Weybridge" Strain of B. anthracis when Produced on a Large Scale.*

Total volume of filtrate per rabbit (ml.)	0.5	0.17	0.1
Rabbits surviving	9	7	2
Rabbits in test	10	12	12

All control animals died.

Production of protective antibodies in the horse.

Hyper-immune serum prepared by injecting animals with cultures of *B. anthracis* of increasing virulence can be shown to afford passive protection in rabbits. Gladstone (1946) demonstrated protective action in serum prepared against concentrated antigen produced *in vitro*, but the potency was of a low order. Alum-precipitated antigen, as described in the present paper, has been used to immunise horses, and the potency of the serum produced was at least equal to that of one kindly supplied by Dr. Stableforth from Weybridge (details will be published later). This latter serum had been prepared by hyper-immunisation of the horse with a living spore vaccine. A comparison of the efficiency of the two types of serum is given in Table V.

TABLE V.—*The Result of Passive Immunity Tests in Rabbits. Immune Horse Serum Given Intravenously and Animals Challenged Intracutaneously 24 Hours later with Approximately 250 A.L.D. of Spores.*

Amount of serum (ml.)	Horses.			"Weybridge" serum.	
	25.	27.	28.		
10	4/4	3/4	4/4	4/4	
3	4/4	1/4	3/4	1/4	
1	1/4	0/4	0/4	0/4	
Normal serum	—	—	—	—	0/4
<i>Nil</i>	—	—	—	—	0/4

Numerator denotes survivors.

DISCUSSION.

Adherence to Pasteurian methods for the prophylaxis of anthrax has been dictated by the fact that no alternative was known. A sterile non-toxic product would have advantages, and this would be particularly true if the material could be produced in bulk in a semi-synthetic medium from a non-virulent strain. Apart from its probable usefulness at least in veterinary medicine, the study of the chemical nature of the antigen involved would be greatly facilitated. The work described has been directed to this end. No comparison has been made of the relative effectiveness of the well-known spore vaccine with the culture filtrate, because it has not seemed possible to devise a test that would give any quantitative estimate of difference in potency. However, the injection of alum-precipitated culture filtrate in two doses gives solid protection against anthrax in rabbits challenged by the intradermal route, and in monkeys challenged by the respiratory route. A lasting immunity seems to develop; monkeys re-challenged by the respiratory route twelve months after immunisation show no evidence of a lower resistance. Furthermore, the material is apparently completely non-toxic.

The advantages of the product for the chemical study of protective antigenic materials have been borne out by experiment, and the results are recorded in the following paper (Strange and Belton, 1954). However, many of the factors controlling the production of this material by culture *in vitro* remain unknown, and are likely to do so until fundamental studies of the metabolic behaviour of *B. anthracis* have been made.

SUMMARY.

Details of a semi-synthetic medium are given, in which certain virulent and non-virulent strains of *B. anthracis* will produce a protective antigen which can be concentrated by lyophilisation or by alum precipitation, and yields a product which is non-toxic.

Large amounts of the antigenic material can be produced with normal laboratory equipment, and rabbits and monkeys have been successfully immunised against anthrax with it.

Hyper-immunisation of horses with the antigen produced a protective serum at least equal in potency to the best that can be obtained by similar immunisation with living spore vaccine.

We wish to thank Dr. D. W. Henderson for introducing this problem and for guidance throughout the work. We are indebted to Dr. B. Record for supervising the freeze-drying of products, and to Mr. S. V. Peacock for carrying out inhalation challenge tests. Our thanks are also due to Mr. I. Whitlock and Mr. B. Eckersley for technical assistance, to Mr. R. Cook for bleeding horses and to Mr. L. Harris for large scale media production.

Acknowledgment is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

REFERENCES.

- BAIL, O.—(1904) *Zbl. Bakt.*, Abt. 1, **36**, 266.
BARTON-WRIGHT, E. C.—(1944) *Biochem. J.*, **38**, 314.

- BREWER, C. R., McCULLOUGH, W. G., MILLS, R. C., ROESSLER, W. G., HERBST, E. J., AND HOWE, A. F.—(1946) *Arch. Biochem.*, **10**, 65.
- CROMARTIE, W. J., WATSON, D. W., BLOOM, W. L., AND HECKLY, R. J.—(1947) *J. infect. Dis.*, **80**, 14.
- DRUETT, H. A., HENDERSON, D. W., PACKMAN, L., AND PEACOCK, S.—(1953) *J. Hyg., Camb.*, **51**, 359.
- GLADSTONE, G. P.—(1946) *Brit. J. exp. Path.*, **27**, 394.—(1948) *Ibid.*, **29**, 379.
- Idem* AND FILDES, P.—(1940) *Ibid.*, **21**, 161.
- STAMP, LORD—(1947) *J. gen. Microbiol.*, **1**, 251.
- STAUB, A. M., AND GRABAR, P.—(1944) *Ann. Inst. Pasteur*, **70**, 16.
- STERNE, M.—(1937) *Onderstepoort J. vet. Sci.*, **8**, 271.
- STRANGE, R. E., AND BELTON, F. C.—(1954) *Brit. J. exp. Path.*, **35**, 153.
- WRIGHT, G. G., HEDBERG, MARY A., AND FEINBERG, R. J.—(1951) *J. exp. Med.*, **93**, 523.
- Idem*, HEDBERG, MARY A., AND SLEIN, J. B.—(1954) *J. Immunol.*, in press.
-