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The Serological Relationship between Purified Preparations of Factors I and II of the Anthrax Toxin Produced *In vivo* and *In vitro*

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SUMMARY: A serological comparison on gel-diffusion plates of purified preparations of factors I and II of the anthrax toxin obtained *in vitro* and *in vivo* indicated that all but one (factor I from *in vitro* sources) of these preparations contained at least two major serological components. At least three serological components were involved in the total system: A (associated with factor I activity); B (associated with factor II activity); C (not associated with toxic activity). These results indicated that no preparation of factor II of the anthrax toxin so far examined was immunologically homogeneous and that the serological connexion between factor I of the anthrax toxin (produced *in vivo*) and the protective factor produced *in vitro* (Smith, 1958) was due to a common component not associated with factor I activity. There was some evidence that the method of measuring immunogenicity in certain culture filtrates of *Bacillus anthracis* by serological precipitation (Thorne & Belton, 1957) is not generally applicable to all products of this organism.

In the previous paper (Stanley, Sargeant & Smith, 1960) the purification of factors I and II of the anthrax toxin from the plasma of guinea-pigs dying of anthrax was described. The factor I preparation contained a small amount of guinea-pig plasma constituents and the factor II preparation was largely guinea-pig α -globulin. An immunogenic preparation from *Bacillus anthracis* was first produced *in vitro* in a defined medium by Wright, Hedberg & Slein (1954) and later in a partially-defined medium by Belton & Strange (1954). Smith, Keppie & Stanley (1955) showed that this non-toxic preparation was serologically related to anthrax toxin; later Smith *et al.* (1956) showed that partially purified material (Strange & Belton, 1954) had factor II activity. Our colleagues Strange & Thorne (1958) carried this purification further and we have shown that their final product also has high factor II activity. Following the production of anthrax toxin *in vitro* by Harris-Smith, Smith & Keppie (1958), C. B. Thorne, D. D. Molnar & R. E. Strange (private communication) found that the other factor of the toxin was produced along with their protective preparation but was adsorbed on the glass filters used to obtain a sterile filtrate. This factor could be removed by eluting the filters with a buffer of high pH value. We confirmed that in relation to the *in vivo* products the adsorbed factor was factor I of the toxin; we have now purified it by a process similar to that described for obtaining factor I from toxic guinea-pig plasma.

The preparations described above all appeared to form one main precipitation line in serological gel-diffusion plates when diffused separately at relatively high concentrations against anthrax antisera in the system used by Thorne & Belton (1957) for the assay of immunizing antigen (Strange & Thorne,

1958; Stanley & Smith, 1958; Sargeant & Smith, 1958). However, when the various preparations were examined more closely by diffusion at high concentrations, by diffusion adjacent to one another, and by diffusion over greater distances with more acute angles between the planes of precipitation, it appeared that three of the four preparations were mixtures of major serological components. Some precipitation lines which appeared to be single in the simple system then appeared to be composite. The present paper describes the serological comparison of these different products and indicates which serological entities are associated with toxicity. These results have some bearing on any general application of the assay for immunogenicity in certain culture filtrates from *Bacillus anthracis* by serological precipitation (Thorne & Belton, 1957).

METHODS

Assays for factors I and II of the anthrax toxin were described by Stanley, Sargeant & Smith (1960).

Fresh anthrax toxin produced in vivo. This was prepared as described by Stanley *et al.* (1960).

Immunizing activity. Active immunity tests were carried out in rabbits as described by Strange & Thorne (1958).

Purified factors I and II of the anthrax toxin produced in vivo. These were the final preparations described by Stanley *et al.* (1960). They were kept at -20° and examined as quickly as possible after preparation.

Purified protective antigen of Strange & Thorne (1958). We are indebted to our colleague R. E. Strange for providing freshly prepared antigen fraction C2 which was fully satisfactory in active immunity tests in rabbits, and in agar diffusion test titrations against anthrax antisera. Fraction C2 was kept at -20° and examined as quickly as possible after preparation. In the assay for factor II activity, a solution containing 100 units antigen/ml. (Strange & Thorne, 1958) had a null point dilution of 1/100, and was therefore about 50 times as active per unit weight as was the preparation of factor II produced *in vivo*.

Purified factor I from toxin produced in vitro. A culture (1 l.) containing crude antigen of *Bacillus anthracis* (Thorne & Belton, 1957; Strange & Thorne, 1958) was passed through a glass filter (6 cm. diameter), which was washed with 0.05 M-phosphate buffer (pH 7; 4×20 ml.) at 0° . On extraction of the filter with ice cold saturated sodium carbonate solution (4 ml.), followed by neutralization of the extract with hydrochloric acid, a solution of crude factor I was obtained. This was fractionated by a modification of the method used to separate factor I produced *in vivo* (to be published). The purified preparation showed no evidence of heterogeneity in the ultracentrifuge at pH 7.4, or on paper electrophoresis at pH 8.6 and 5.0. Its solution (0.2%) had a factor I null point at dilution 1/32,000.

Serological precipitation in gels. Unless otherwise stated, the Ouchterlony gel-diffusion procedure was adopted, with the system of wells devised by Burrows & Bacon (1958) and used by Thorne & Belton (1957). Samples

(0.1 ml.) were diffused against anthrax antisera (0.1 ml.) which had been placed in the wells 24 hr. before the antigen. Diffusion was at 0–2° in plates buffered at pH 7.3. Three antisera were used.

Antiserum H. 533 was serum from a horse hyperimmunized with live spores of the 'Sterne' strain of *Bacillus anthracis*. Since this strain produces the anthrax toxin in a serum medium (Harris-Smith *et al.* 1958) this antiserum should detect both factors of the anthrax toxin.

Antiserum H. 25. This was serum from a horse hyperimmunized with the crude alum-precipitate of culture filtrates from the 'Sterne' strain of *Bacillus anthracis*. Since this serum was prepared from the protective filtrate it follows, from what has been said in the introduction, that it should detect factor II of the anthrax toxin but should not react with factor I since the latter factor is removed by filtration of the culture. On the other hand, this antiserum to the crude culture filtrate would be more likely to detect impurities of degradation products of the filtrate factor than would antiserum to live spores (antiserum H. 533).

Antiserum R-1. This was serum from a rabbit immunized with the final preparation of *in vivo* factor I isolated from toxic guinea-pig plasma (Stanley *et al.* 1960).

RESULTS

To facilitate the description of results the antigenic factor I produced *in vivo*, factor II produced *in vivo*, factor I produced *in vitro* and the protective antigen produced *in vitro* (having high factor II activity) will be called fractions I, II, 1 and 2, respectively. When a preparation is described as toxic this means toxic when mixed with the appropriate complementary factor.

Fig. 1. Serological precipitation lines formed by (a) a mixture (M) consisting of fraction 1 (5 μ g.), fraction 2 (5 μ g.) and fraction II (100 μ g.) with antisera H. 533 and H. 25; (b) fraction I (40 μ g.), fraction II (100 μ g.), fraction 1 (5 μ g.) and fraction 2 (5 μ g.) diffused adjacent to mixture M against antiserum H. 533; (c) as (b) but with antiserum H. 25.

Gel-diffusion precipitation when preparations of the two toxic factors were diffused side by side with a mixture (M) of fractions II, 1 and 2

When a mixture M of fractions II, 1 and 2 was diffused against antisera H. 533 and H. 25 the precipitation lines shown in Fig. 1a were formed. There were at least three serological components: A (nearest the antigen well)

precipitated by antiserum H. 533 and not by antiserum H. 25; B precipitated by both antisera; C (nearest the antiserum wells) precipitated by antiserum H. 25 and only weakly by antiserum H. 533.

The four fractions, I, II, 1 and 2 were then diffused adjacent to the mixture M, against each antiserum separately (see Figs. 1*b* and 1*c*). Although no preparation appeared to form more than one line against either antiserum it was clear that fraction I contained components A and B, in addition to a third component detected by antiserum H. 533 and giving rise to a spur in Fig. 1*b*; fraction II contained component C; fraction 1 contained component A; fraction 2 contained components B and C. When the four preparations were diffused adjacent to fresh anthrax toxin produced *in vivo*, the latter was shown to contain serological components A, B and C, among others. In view of these results of diffusion in a simple system, each preparation was examined critically for further evidence of serological heterogeneity.

Evidence that fraction II contained a mixture of two serological components (B and C)

On a weight basis, the factor II activity of fraction II (which was mainly guinea-pig α -globulin) was much lower than the activity of fraction 2. When more concentrated solutions of fraction II (c. 1000 μ g./well and having factor II activities similar to those of the solutions of fraction 2 used for gel diffusion) were diffused on plates a second precipitation line was formed. This proved to be due to serological component B, when fraction II was diffused adjacent to mixture M against antiserum H. 25 (see Fig. 2*a*).

Evidence that fraction 2 contained serological components B and C

Comparison of fraction 2 with fraction II by diffusion against antiserum H. 25, using a larger antiserum well, a greater distance between the wells and an arrangement which produced a more acute angle between planes of precipitation. The two fractions 2 and II were compared side by side on a diffusion plate in which three wells of diameter 9 mm. were arranged in the form of an equilateral triangle (distance between centres of wells 19 mm.). The amount of fraction II used (200 μ g.) was such that lines B and C were both clearly visible. The results of diffusion in this system against antiserum H. 25 (Fig. 2*b*) showed that both B and C lines were present in fraction 2 (10 μ g.), but the ratio of B to C was greater than in fraction II.

Displacement of line C formed by fraction 2 from its position overlying line B, by adding more of component C (in fraction II). The intensity of the C line formed by fraction II (100 μ g.; see Fig. 1*c*) was less than the intensity of the C line formed by the mixture M. The latter contained the same amount of fraction II (100 μ g.), fraction 1 (5 μ g.) which did not react with antiserum H. 25, and fraction 2 (5 μ g.). Hence fraction 2 must have contributed to C line formed by mixture M. This conclusion was supported by the results shown in Fig. 2*a* where a much larger quantity of fraction II (1000 μ g.) was required to form a C line of comparable intensity to that produced by mixture

M. The second line (line B) formed by mixture M against antiserum H. 25 (Fig. 1c) was due to fraction 2 since neither fraction 1 (5 μ g.) nor fraction II (100 μ g.) formed this line. These results were confirmed by adding fraction II (100 μ g.) to fraction 2 (5 μ g.) so that two lines were formed with antiserum H. 25, and demonstrating by titration that the dilution of the mixture which first formed the C line was 4 times higher than the corresponding dilution of fraction II (100 μ g.) when diffused alone.

Displacement of line B formed by fraction 2 from its position overlying line C by decreasing the proportion of component B, with an antiserum specific to this component. When rabbit antiserum (R-1) produced against fraction I (see Methods) was added to fraction II 1 hr. before placing fraction II in gel-diffusion plates against antiserum H. 25, the line corresponding to component B did not appear. This rabbit antiserum R-1 was added to fraction 2 in increasing amounts and after an hour the resultant mixtures were diffused

Fig. 2. Serological precipitation lines formed against antiserum H. 25 by: (a) fraction II (1000 μ g.) and a mixture (M) of fraction 1 (5 μ g.), fraction 2 (5 μ g.) and fraction II (100 μ g.); (b) fraction II (2000 μ g.) and fraction 2 (10 μ g.) in a system in which the wells (9 mm. diameter) were arranged in the form of an equilateral triangle (centres of wells 19 mm. apart); (c) fraction 2 (5 μ g.) alone, in the end wells, and after being mixed with 0.05 ml. (well X), 0.025 ml. (well Y) and 0.012 ml. (well Z) of antiserum R-1, 1 hr. before use.

against antiserum H. 25. The results (Fig. 2c) showed that as the amount of rabbit antiserum added to fraction 2 was increased, the two superimposed lines usually formed by fraction 2 separated, and that one was almost eliminated by the largest quantity of antiserum R-1. Appropriate controls showed that normal rabbit serum did not produce this effect; additional experiments, in which mixtures of fraction 2 and antiserum R-1 were flanked by fraction II at high concentration, showed that the serological component decreased in quantity and eventually removed was B and that the remaining component was C.

Progressive destruction of serological component B, but not C, by incubation with in vivo toxin. The evidence (see below) supports the other findings that fraction 2 was a mixture of components B and C. It is, however, possible that under these conditions a rapid split of fraction 2 into components B and C preceded the destruction of B, or that some C was formed from B.

Evidence that factor II activity was associated with serological component B and that fraction I contains this component in a non-toxic, but antigenic, form

Removal of serological component B and factor II activity by treating fraction II with an antiserum (R-1) to fraction I. Serological component B was common to fractions I and II, but serological component C was present only in fraction II (Fig. 1). Reaction of fraction II with γ -globulin from a rabbit antiserum to fraction I (R-1) removed factor II activity and serological component B, but left component C intact.

γ -Globulin was separated from rabbit antiserum to fraction I (R-1) by the method of Deutsch (1952) and dissolved in saline at pH 7.4. This solution was added to crude factor II (Stanley *et al.* 1960) in slight excess of the amount required to suppress serological component B (11 ml. crude factor II required 8 ml. γ -globulin solution). This amount was determined by titration in a diffusion plate with antiserum H. 25. The crude factor II was then fractionated as described by Stanley *et al.* (1960) to obtain the product after chromatography on a diethylaminoethyl cellulose column. A mixture of crude factor II (11 ml.) and saline (8 ml.) was processed similarly as a control.

The dialysed and freeze-dried products obtained from the γ -globulin-treated and control mixtures weighed 31 and 38 mg., respectively. The treated product (4 mg.) showed no factor II activity, whereas the control material had a factor II null point at 50 μ g. When each product was titrated on a gel-diffusion plate against antiserum H. 25, serological component B was detected in the treated product (8 mg.) although it was still precipitable from the control (0.5 mg.); in both cases the C line was still detectable at 60 μ g.

It was important to be sure that the absence of factor II activity from the treated sample was not due to the presence of antibody to factor I (since antiserum R-1 was produced against fraction I) which neutralized the factor I used in the assay of factor II. For this reason the immune γ -globulin was added to crude factor II, because the subsequent fractionation of factor II was known to remove excess γ -globulin. The absence of antibody to factor I from the final product was tested by including it in a toxic mixture containing an excess of factor II, i.e. a mixture the toxicity of which was limited by its factor I content. The size of the skin reaction was the same as that produced by the toxic mixture alone.

Preferential destruction of serological component B and factor II activity in fraction 2 by incubation with toxin produced in vivo. Crude toxin produced *in vivo* contained a system which destroyed factor II activity at 37°. The serological precipitation of a mixture of fraction 2 and this toxin was studied after incubation. A solution consisting of fraction 2 (1 ml., factor II null point 1/640) toxin produced *in vivo* (1 ml., null point 1/80) and 0.05M-tris buffer (pH 8; 8 ml.), had a factor II null point of 1/128. A sample was kept at -20° and the remainder incubated at 37°. Samples removed at 3, 8, 24 and 48 hr. had factor II null points of 1/128, 1/64, 1/8 and 1/1, respectively. The results obtained on precipitation of these samples (undiluted) in plates with antiserum H. 25 are shown in Fig. 3. The usual composite line was present

initially and when titrated was just visible at a dilution of 1/64. After 8 and 8 hr. two lines were seen, both of which joined smoothly into the line formed by the original sample. With the mixture M (see above) as a control, the line nearer the antigen wall (when titrated, just visible at 1/32 of both samples) was shown to be the B line, and that nearer the antiserum well was shown to be the C line (also just visible at 1/32 of both samples). After 24 hr. the B line was much weaker (just visible at 1/2) and after 48 hr. it could not be detected with antiserum H. 25; but the C line was still present in unchanged intensity (just visible at 1/32). Antiserum H. 583 formed one line (the B line) with the original sample (just visible at a dilution of 1/128). After the sample had been incubated for 48 hr. at 37° this line was not detectable at a dilution higher than 1/2. In a control experiment the toxin produced *in vivo*, at the concentration used in the original mixture, did not give a line with either antiserum. Active immunity tests, by the procedure of Strange & Thorne (1958), on the original mixture and on the product after heating for 48 hr., showed that a considerable degree of protective activity survived this treatment. Table 1 shows the results of these immunity tests compared with the factor II activities and serological precipitation data for the two samples.

Fig. 3. Serological precipitation lines formed against antiserum H. 25 by mixtures of fraction 2 (5 μ g.) and anthrax toxic plasma (0.0025 ml.) after incubation for 0, 8, 24 and 48 hr. with a mixture M (see Fig. 1) as a marker of B and C lines.

These results suggested that: (a) factor II activity was associated with the B line and not with the C line; (b) fraction 2 could lose its factor II activity and still retain appreciable protective power; (c) titration of these solutions with antiserum H 583 in gel-diffusion plates was not a measure of their protective power and spore (H. 583)—and antigen (H. 25)—antisera could produce widely differing titration results with the same sample (cf. Thorne & Belton, 1957; Strange & Thorne, 1958).

Examination of fraction 1 for the presence of more than one serological component

In the short diffusion system fraction 1 (5 μ g.) formed a single line (A) with antiserum H. 583 and this line did not appear to be composite when fraction 1 was flanked by a mixture of fractions II, 2 and 1 (Fig. 1*b*) or by fraction I. When allowed to diffuse over a greater distance (10 mm.) fraction 1 (10 μ g.) still did not form more than one line. This amount of fraction 1 did not form precipitation lines with antiserum H. 25. However, when 50 μ g. of fraction 1 were placed in the short diffusion system a faint second line was formed against antiserum H. 583 and two faint lines were formed against antiserum H. 25.

These additional lines were probably not associated with factor I activity since they were also formed by far smaller amounts of some relatively inactive fractions discarded during the purification of factor I.

Table 1. *Comparison of factor II activity, serological precipitation and protective activity of fraction 2, before and after incubation with a small amount of anthrax toxin produced in vivo*

	Initial	After 48 hr. at 37°																		
Factor II null point	1/128	Not active undiluted																		
Gel-diffusion precipitation*																				
(a) With antiserum H. 25																				
Line B	1/64†	Not formed undiluted																		
Line C	1/64†	1/32																		
(b) With antiserum H. 588																				
Line B	1/128	1/2																		
Protective activity in rabbits. Total amount of fraction 2 injected with normal horse serum‡	<table> <tr> <th></th><th>No. survivors</th><th>No. treated</th></tr> <tr> <td>(µg.)</td><td></td><td></td></tr> <tr> <td>28</td><td>18/19</td><td>10/20</td></tr> <tr> <td>2.8</td><td>18/19</td><td>7/20</td></tr> <tr> <td>0.28</td><td>2/19</td><td>1/20</td></tr> <tr> <td>Nil</td><td>0/20</td><td>—</td></tr> </table>			No. survivors	No. treated	(µg.)			28	18/19	10/20	2.8	18/19	7/20	0.28	2/19	1/20	Nil	0/20	—
	No. survivors	No. treated																		
(µg.)																				
28	18/19	10/20																		
2.8	18/19	7/20																		
0.28	2/19	1/20																		
Nil	0/20	—																		

Details of the initial solution are given in the text.

* The dilution quoted in each case is the greatest at which a line was still formed.

† After heating for 1 hr., the lines were sufficiently apart to see individual titrations.

‡ The test was carried out as described by Strange & Thorne (1958).

DISCUSSION

A serological comparison on gel-diffusion plates of the purified preparations of factors I and II of the anthrax toxin obtained *in vitro* and *in vivo* indicated that all but one of these contained at least two major serological components. Not less than three serological entities, designated A, B and C, were involved in the total system. Component A appeared to be associated with factor I activity. Component B appeared to be associated with factor II activity, although it was present in one preparation having no factor II activity. Component C did not appear to be associated with toxic activity. Components A and B were precipitated well and component C only weakly by an anthrax antiserum against live spores (antiserum H. 588). Components B and C were precipitated well and component A not at all by an anthrax antiserum against crude antigen produced *in vitro* (Belton & Strange, 1954).

The behaviour of the various preparations was as follows.

(1) The purified preparation of factor I produced *in vitro* contained the single major serological component A.

(2) The purified preparation of the protective antigen produced *in vitro* (Strange & Thorne 1958) contained a mixture of serological component B, associated with factor II activity, and the non-toxic component C. These components appeared to be present in such concentrations that the two precipitation lines were superimposed. This preparation did not contain component A and the apparent connexion between *in vivo* factor I and the purified antigen (Smith, 1958) was due to the presence in each of component B (see below).

(3) The purified preparation of factor I produced *in vivo* (Stanley *et al.* 1960) contained, in addition to serological component A (associated with factor I activity), some component B despite the fact that it had no factor II activity. Since our antiserum to this preparation neutralized factor II activity, it was possible that component B was here present in a non-toxic but antigenic form. In addition to components A and B this preparation also contained another serological component which was not present in the other three preparations.

(4) The partially purified preparation of factor II produced *in vivo* (Stanley *et al.* 1960) contained a mixture of serological components B (associated with factor II activity) and the non-toxic component C, as well as much guinea-pig α -globulin. The proportion of component B to component C was less than that in the preparation from the culture *in vitro*.

It is possible that one or more of the various preparations which appeared to contain mixtures of antigens contained only single antigens and that evidence for two or more serological components was due to decomposition during the diffusion and precipitation. We believe this to be unlikely, however, in view of the low temperature and neutral pH values at which all experiments were carried out. At the moment it is impossible to estimate the proportions of the three serological components in these mixtures, since the relationship between weight and ability to produce precipitation lines is unknown, at least for components B and C.

It is clear from these results that the method of measuring immunogenicity in certain culture filtrates described by Thorne & Belton (1957) is not generally applicable to all products of *Bacillus anthracis*. In our experiments, line titres of test preparations against 'spore' antiserum (H. 538) and 'antigen' antiserum (H. 25) did not agree with one another, or with the results of active immunity tests. Furthermore, as a criterion of serological purity, the production of a single precipitation line after diffusion alone in the simple system described by Thorne & Belton (1957) should be supplemented by experiments in which diffusion takes place from wells adjacent to those containing likely impurities, and possibly over a greater distance, in an arrangement of wells which will produce a more acute angle between planes of precipitation.

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REFERENCES

- BELTON, F. C. & STRANGE, R. E. (1954). Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*: medium and methods of production. *Brit. J. exp. Path.* **35**, 144.
- BURROWS, T. W. & BACON, G. A. (1958). The effects of loss of different virulence determinants on the virulence and immunogenicity of strains of *Pasteurella pestis*. *Brit. J. exp. Path.* **39**, 278.
- DEUTSCH, H. F. (1952). Separation of antibody-active proteins from various animal sera by ethanol fractionation techniques. *Meth. med. Res.* **5**, 284.
- HARRIS-SMITH, P. W., SMITH, H. & KEPPIE, J. (1958). Production *in vitro* of the toxin of *Bacillus anthracis* previously recognized *in vivo*. *J. gen. Microbiol.* **19**, 91.
- SARGEANT, K. & SMITH, H. (1958). Purification of factor II of the anthrax toxin produced *in vivo* using cellulose ion-exchange columns. *Biochem. J.* **69**, 55 f.
- SMITH, H. (1958). The basis of immunity to anthrax. *Proc. roy. Soc. Med.* **51**, 375.
- SMITH, H. & GALLOP, R. C. (1956). The chemical basis of the virulence of *Bacillus anthracis*. VI. An extracellular immunising aggressin isolated from exudates of infected guinea pigs. *Brit. J. exp. Path.* **37**, 144.
- SMITH, H., KEPPIE, J. & STANLEY, J. L. (1955). The chemical basis of the virulence of *Bacillus anthracis*. V. The specific toxin produced by *B. anthracis in vivo*. *Brit. J. exp. Path.* **36**, 460.
- SMITH, H., TEMPEST, D. W., STANLEY, J. L., HARRIS-SMITH, P. W. & GALLOP, R. C. (1956). The chemical basis of the virulence of *Bacillus anthracis*. VII. Two components of the anthrax toxin: their relationship to known immunising aggressins. *Brit. J. exp. Path.* **37**, 263.
- STANLEY, J. L. & SMITH, H. (1958). Purification of factor I of the anthrax toxin produced *in vivo*. *Biochem. J.* **69**, 55 f.
- STANLEY, J. L., SARGEANT, K. & SMITH, H. (1960). Purification of factors I and II of the anthrax toxin produced *in vivo*. *J. gen. Microbiol.* **22**, 206.
- STRANGE, R. E. & BELTON, F. C. (1954). Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*: Purification and chemistry of the antigen. *Brit. J. exp. Path.* **35**, 158.
- STRANGE, R. E. & THORNE, C. B. (1958). Further purification studies on the protective antigen of *Bacillus anthracis* produced *in vitro*. *J. Bact.* **76**, 192.
- THORNE, C. B. & BELTON, F. C. (1957). An agar diffusion method for titrating *Bacillus anthracis* immunizing antigen and its application to a study of antigen production. *J. gen. Microbiol.* **17**, 505.
- WRIGHT, G. C., HEDBERG, M. A. & SLEIN, J. B. (1954). Studies on immunity in anthrax. III. Elaboration of a protective antigen in a chemically defined, non-protein medium. *J. Immunol.* **72**, 268.

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