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The Capsular Substance of *Bacillus anthracis*

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Iványovics and his colleagues (Iványovics & Erdös, 1937; Brückner & Iványovics, 1937; Iványovics & Brückner, 1937, 1938), following up the earlier work of others (Kramar, 1922; Tomcsik & Bodon, 1934), isolated the capsular substance from capsulated strains of *Bacillus anthracis* and some related organisms in a state approaching purity; the material was shown to have the properties of a hapten, giving precipitates with anti-sera to capsulated strains of *B. anthracis* but failing to induce antibody formation or to confer protection against infection with *B. anthracis* when injected into animals. On hydrolysis, the capsular substance gave good yields of *d*-(−)-glutamic acid, enantiomorphous with the *l*-(+)-acid normally encountered in proteins, and Iványovics & Brückner (1937, 1938) concluded that it was a polypeptide built up solely of *d*-(−)-glutamic acid residues. The work described in the present paper had as its immediate object the elucidation of the detailed chemical structure of this material.

Our work has mostly been carried out with two capsulated strains of *B. anthracis*, viz. 'Vollum', a highly virulent strain originally obtained from Dr R. L. Vollum of the Sir William Dunn Institute of Pathology, Oxford, and 'HM', a non-sporing variant of low virulence obtained by Dr Bruce White, F.R.S., of the National Institute for Medical Research, Hampstead, by growing 'Vollum' in Sclavo serum (see Appendix).

Isolation of crude capsular material

After some preliminary trials the two following methods were found convenient for the isolation of the crude capsular substance:

(*a*) *Cultures grown on agar* are reaped, suspended in water and killed by autoclaving (45 min. at 115°). The autoclaved suspension is strongly acidified (HCl) and treated with 3 vol. of ethanol; after removing bacterial debris by filtration or centrifugation, the crude capsular substance is precipitated from the filtrate by bringing to pH 8–9 with NaOH. (This procedure is a development of a method, fully described in the Appendix, originally devised by Dr Bruce White.)

(*b*) *Cultures grown in liquid media* are autoclaved and centrifuged or filtered. The filtrate is treated with 10% $CuSO_4$ solution and the precipitated copper salt collected by centrifugation; the washed salt is dissolved in acid and decomposed with H_2S. The filtrate from the CuS is adjusted to pH 8–9 and precipitated with ethanol (3 vol.).

Crude materials so prepared from the Vollum and HM strains (and also from another strain, '99', of American origin) were serologically active, giving precipitates with the appropriate anti-serum at dilutions down to 1 in 10^7. These crude preparations give extremely viscous solutions in water, and the best of them probably approximate very closely to the capsular substance as it occurs attached to the organism.

Further purification of capsular substance

Further purification of the capsular substance without extensive degradation is not easy. Dialysis in acid solution is not wholly satisfactory since the molecular weight, calculated from the amino-nitrogen content on the assumption that each molecule contains only one free amino-group, decreases considerably during the process, e.g. the amino-nitrogen content of a crude preparation was doubled by keeping at 25° for 48 hr. in solution at pH 2. The most satisfactory method of purification is by way of the silver salt, which is precipitated

when $AgNO_3$ is added to a solution of the crude capsular substance. The silver salt so obtained is washed, suspended in water and shaken with an excess of the appropriate chloride (NaCl for the preparation of the sodium salt, HCl for the free acid, etc.); AgCl is removed by filtration and the filtrate is dialyzed. In the preparation of the sodium salt dialysis is first carried out, until all chloride ion has been removed, against distilled water with NaOH added to bring the pH to 8–9; the product is then dialyzed against distilled water until its pH falls to 7. In the preparation of the free acid the filtrate is simply dialyzed against distilled water until chloride-free; some degradation under these necessarily acid conditions is unavoidable and the dialysis should not be unnecessarily prolonged. The dialyzed solutions are concentrated to small bulk *in vacuo* at room temperature and finally dried, *in vacuo* over P_2O_5, from the frozen state. The materials so obtained are brittle glasses which dissolve in water, with preliminary swelling, to give viscous solutions; the purified materials are about twice as active serologically as the crude products.

Analytical data

Analytical data on various preparations obtained in this way are given in Tables 1 and 2. These analytical figures are all in agreement with those calculated for a glutamic acid polypeptide containing water not removed under the necessarily mild conditions of drying (P_2O_5 at room temperature); both sulphur and phosphorus are absent from our preparations. The products all give the pink staining reaction with Loeffler's methylene blue shown by the native capsular substance, and even the most highly degraded material (H 213 B) was serologically active (precipitin reaction with anti-capsular serum down to 1 in 2×10^7). The molecular weights are calculated on the assumption that the molecule contains only one free amino-group; the calculations would be invalidated by pyrrolidone ring formation, but the absence of this disturbing factor is rendered probable by the qualitative observation that the viscosity of the aqueous solution generally runs parallel with the calculated molecular weight. The variations in molecular weight seem to depend on the time of contact with acid. Nearly

Table 1. *Analytical data for the capsular substance (sodium salt) of* B. anthracis

Prep. no.	C %	H %	N %	Na %	NH_2-N* %	C : H : N : Na ratio	Mol. wt. (from N/NH_2-N)	No. of glutamic residues (from N/NH_2-N)	Remarks
H 228 B	36·4	4·9	8·3	13·3	0·024	5·1 : 8·3 : 1·0 : 1·0	53,000	350	From HM strain
F 9	34·6	5·8	8·0	13·0	0·040	5·05 : 10·2 : 1·0 : 1·0	30,000	200	From HM strain
C 187 A	34·45	4·8	8·1	13·6	0·045	5·0 : 8·3 : 1·0 : 1·0	27,000	180	From HM strain
C 187 B	32·7	4·8	8·5	12·75	0·061	4·5 : 8·0 : 1·0 : 0·9	21,000	140	From HM strain
F 6	33·35	4·9	7·6	15·0	0·076	5·1 : 9·1 : 1·0 : 1·2	15,000	100	From Vollum strain
H 228 A	37·1	5·4	8·4	11·3	0·085	5·0 : 9·0 : 1·0 : 0·8	15,000	100	From Vollum strain
F 4 A	37·95	5·9	8·4	11·9	0·092	5·3 : 9·8 : 1·0 : 0·9	14,000	90	From HM strain (purified through Cu salt)
—	39·7	4·0	9·3	15·2	0	5 : 6 : 1 : 1	∞	∞	Theoretical for anhydrous Na salt $(C_5H_6O_3NNa)_\infty$

* Van Slyke; 4 min. reaction time. Longer reaction times give higher values owing to degradation (see p. 300). For this reason all the calculated molecular weights and chain lengths are *minimal* values.

Table 2. *Analytical data for the capsular substance (free acid) of* B. anthracis

Prep. no.	C %	H %	N %	Ash %	NH_2-N* %	C : H : N ratio	Mol. wt. (from N/NH_2-N)	No. of glutamic residues (from N/NH_2-N)	Equiv.	Remarks
H 230 B	44·0	6·1	10·3	1·2	0·064	5·0 : 8·3 : 1·0	21,000	160	145	From HM strain
F 2	44·2	6·1	11·0	0·6	0·116	4·7 : 7·8 : 1·0	12,000	95	140	From HM strain
F 5	—	—	9·8	1·05	0·126	—	10,000	80	141	From Vollum strain
F 2 A	44·95	6·2	9·8	2·6	0·145	5·3 : 8·9 : 1·0	8,700	70	137	From HM strain
H 213 B	38·2	6·5	9·5	2·5	0·280	4·7 : 9·5 : 1·0	4,500	35	—	From HM strain
—	—	—	9·5	2·9	0·20	—	6,000	48	166	Ivánovics & Brückner (1937). Mean values
—	46·4	5·4	10·9	0	0	5 : 7 : 1	∞	∞	129	Theoretical for $(C_5H_7O_3N)_\infty$

* Van Slyke; 4 min. reaction time. Longer reaction times give higher values owing to degradation (see p. 300). For this reason all the calculated molecular weights and chain lengths are *minimal* values.

all our preparations are less degraded than those of Ivánovics & Brückner (1937); the high molecular weight H 228 B is comparable with that of serum albumin (67,000) and higher than those of accepted proteins such as ovalbumin (41,000) and insulin (35,000), and the capsular substance might legitimately be regarded as a protein of particularly simple composition.

The amino-acid composition of the capsular substance

Having thus developed satisfactory procedures for the isolation and purification of the capsular substance, we turned our attention to the problem of its amino-acid composition. Ivánovics & Brückner (1937) obtained 86 and 70 % yields of *d*-(−)-glutamic acid by the hydrolysis of capsular material obtained from *B. mesentericus* and *B. anthracis* respectively; Bovarnick (1942) reported that 91–97 % of the total nitrogen of capsular material from *B. subtilis* was present as glutamic acid, but, since his material was only obtained in solution, these results can only be accepted with reserve.

It is an easy matter to isolate *d*-glutamic acid from the capsular substance (a convenient preparative procedure is described in the experimental section), and there can be no doubt that *d*-glutamic acid is the major constituent of the capsular substance; it is important, however, to demonstrate as completely as possible the absence of any other amino-acids.

Exclusion of amino-acids other than glutamic acid. We therefore attempted to separate dibasic amino-acids quantitatively from monobasic amino-acids by means of amide-exchanging synthetic resins (Cannan, 1944) with a view to applying the method to hydrolysates of the capsular substance; we were able to effect a separation with both Amberlite IR-4 (as used by Cannan) and De-acidite B (from The Permutit Co.), but the results were not wholly satisfactory, the separation being only partial and the resins themselves yielding variable and not inappreciable amounts of nitrogen under the conditions of the separation. We therefore turned to the use of partition chromatography (Martin & Synge, 1941; Gordon, Martin & Synge, 1943). Using the procedure of these workers, almost identical yields of glutamic acid (42·5 and 44·5 % respectively) were obtained from hydrolyzed capsular material and authentic glutamic acid subjected to the same experimental procedure; this experiment showed conclusively that the following amino-acids are absent from the capsular substance: phenylalanine, leucine, isoleucine, norleucine, tryptophan, valine, norvaline, methionine, proline, alanine and tyrosine. Cystine, too, must be absent since the capsular substance contains no sulphur.

Estimation of glutamic acid content. From the observed band-rate ($R = 0{\cdot}12$–$0{\cdot}14$) of acetylglutamic acid in 17 % butanol-chloroform, it is a simple matter to calculate the partition coefficient between this solvent and water (cf. Martin & Synge, 1941); the value so obtained ($\alpha = 45$) explained our low recovery of glutamic acid. The recovery to be expected with this unfavourable partition coefficient, using the volumes of solvent recommended by Gordon *et al.* (1943), is about 50 %; we found 40–45 %, and the discrepancy may be due to the formation of some neutral material (possibly 5-acetyl-pyrrolidine) during the acetylation procedure. Clearly the method of Gordon, Martin & Synge is not applicable without modification to glutamic acid and other amino-acids whose acetyl-derivatives have such low band-rates. The difficulty can be overcome by using acetic anhydride in boiling water (cf. Behr & Clarke, 1932) for the acetylation; the acetylation mixture can then be evaporated and the acetyl-amino-acids transferred to the column without the necessity of extracting from aqueous solution. This procedure, which may prove useful for other amino-acids, is not completely satisfactory for glutamic acid owing to the formation of a mixture of acetylglutamic acid and pyrrolidone-carboxylic acid.

We finally decided to convert the glutamic acid into pyrrolidone-carboxylic acid. Wilson & Cannan (1937) have worked out the conditions of temperature and pH necessary for rapid and complete cyclization, and this conversion has been employed by various workers as a basis for methods of estimating glutamic acid; thus Olcott (1944) measured the decrease in amino-nitrogen. Such methods are non-specific, but a specific method results if the pyrrolidone-carboxylic acid formed is estimated by partition chromatography; we have worked out such a method which has given excellent results with the capsular substance and which is now being applied to other proteins. The evaporated protein hydrolysate (a mixture of amino-acid hydrochlorides) is treated with an amount of 0·1 N-NaOH equivalent to the combined HCl present; the solution is brought to pH 2·0 with acetic acid, heated to 130° for 4 hr. in a sealed tube and then evaporated to dryness in a vacuum desiccator over KOH. The pyrrolidone-carboxylic acid in the evaporated product is transferred to a silica-gel column with 17 % butanol-chloroform, giving a band ($R = c.\ 0{\cdot}3$) which is eluted and titrated. The residue remaining after transference of the pyrrolidone-carboxylic acid to the column contains the other amino-acids, which can then be estimated by acetylation and partition chromatography. Application of this procedure to authentic *l*-(+)-glutamic acid hydrochloride gave a mean recovery of 95·6 % as pyrrolidone-carboxylic acid; in what follows the appropriate correction factor (1·045) has been applied to determinations of glutamic acid by this method. The method is

rapid and convenient; it is easily applicable to 20 mg. of material, and about 0·2 mg. of glutamic acid can readily be detected and estimated in this amount of material if a good sample of silica gel is used.

The capsular substance composed of d-(−)-glutamic acid units. Two preparations of capsular substance (one from the HM strain, the other from the Vollum strain) were hydrolyzed with HCl. The analytical data obtained for the evaporated hydrolysates are

Table 3. *Analytical data on capsular substance hydrolysates*

	F9 (from HM) (%)	H228A (from Vollum) strain Exp. I (%)	Exp. II (%)
Total N	8·21	8·46	8·20
Amino-N	8·12	8·22	7·92
Amide-N	0·00	0·15	0·19
Glutamic acid-N	8·06; 8·26	8·22	8·00

given in Table 3. The residual materials, after transfer of the pyrrolidone-carboxylic acid to the column, were acetylated with acetic anhydride in boiling water and subjected to partition chromatography. Only two very small bands ($R=0·29$ and $R=0·12$) were detected, and even these were so small that they disappeared before reaching the bottom of the column; their band rates are consistent with pyrrolidone-carboxylic acid and acetylglutamic acid derived from uncyclized glutamic acid. The absence of basic amino-acids and of hydroxyproline was confirmed by the failure of either hydrolysate to give precipitates with phosphotungstic acid or Reinecke's salt. Of all the other amino-acids known to occur in proteins, only glycine ($R=0·15$ for acetylglycine) and aspartic acid ($R=0·16$ for acetylaspartic acid) would give bands moving at rates between $R=0·1$ and $R=0·3$; the presence of minute traces of one or both of these amino-acids has not been rigorously excluded. However, the glutamic acid estimations account for 99 % of the non-amide nitrogen; the discrepancy is well within the estimated experimental error, and it may confidently be concluded that the capsular substance from both the HM and Vollum strains of *B. anthracis* is built up solely from d-(−)-glutamic acid units.

The structure of the capsular substance

The ready fission of the capsular substance by acid suggested that the glutamic acid residues were joined together, in part at least, by linkages more susceptible to acid hydrolysis than normal α-peptide linkages; further evidence for this view was obtained from Van Slyke amino-nitrogen determinations. It was noticed that the apparent amino-nitrogen content increased with increasing reaction time; in Fig. 1 the apparent molecular weight, calculated from the amino-nitrogen values, is plotted against the reaction time in the Van Slyke apparatus. It will be seen that the effect of varying the reaction time is greatest with specimens of high molecular

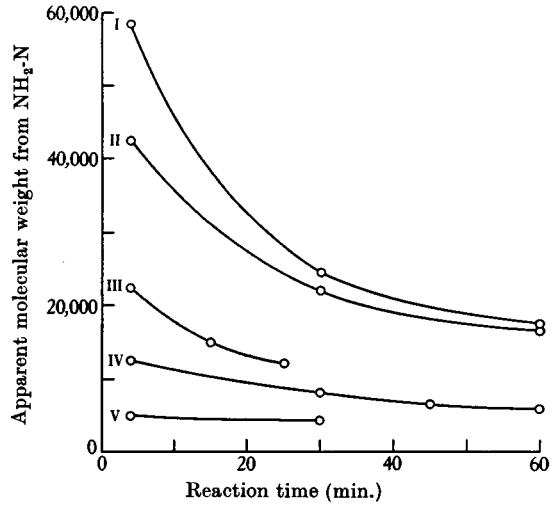

Fig. 1. Effect of varying Van Slyke reaction times on apparent mol.wt. of capsular substance as calculated from amino-nitrogen content. Curve I, H228B; curve II, F9; curve III, H215A; curve IV, F4A; curve V, H213B.

weight and that the apparent molecular weight approaches a limiting value. It is known that glutamine and its *N*-alkyl derivatives yield 2 mol. of nitrogen in the Van Slyke procedure (Chibnall & Westall, 1932; Lichtenstein, 1942), and that glutathione, a γ-peptide of glutamic acid, liberates part of its peptide nitrogen under these conditions; we interpret our Van Slyke results as indicating that, in addition to the free amino-group, the capsular substance also contains γ-peptide groupings which liberate their nitrogen, under the Van Slyke conditions, rather more slowly. The molecular weights given in Tables 1 and 2 are based on amino-nitrogen values obtained with a reaction time of 4 min. and are probably too low owing to some reaction of γ-peptide nitrogen during this period; the error will be greatest in the case of the materials of highest molecular weight. It is noteworthy that one specimen, H213B, of apparent mol. wt. 5000, showed only a slight increase in amino-nitrogen with increased reaction time; this specimen probably represents a final degradation product with only α-peptide linkages, all the readily broken γ-linkages having been hydrolyzed by acid during the preparation. It is probable that Ivánovics' materials of mol. wt. 6000 resemble H213B in this respect.

In any polypeptide of glutamic acid made up of $(m+n+1)$ glutamic acid residues linked together

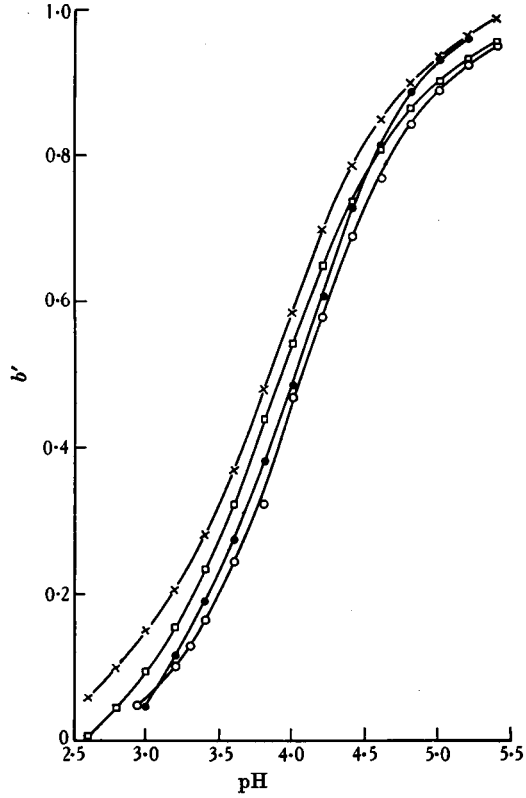

Fig. 2. Titration curves for capsular substance in water. ×——× H228B. ○——○ H230B. □——□ H228A. ●——● F9.

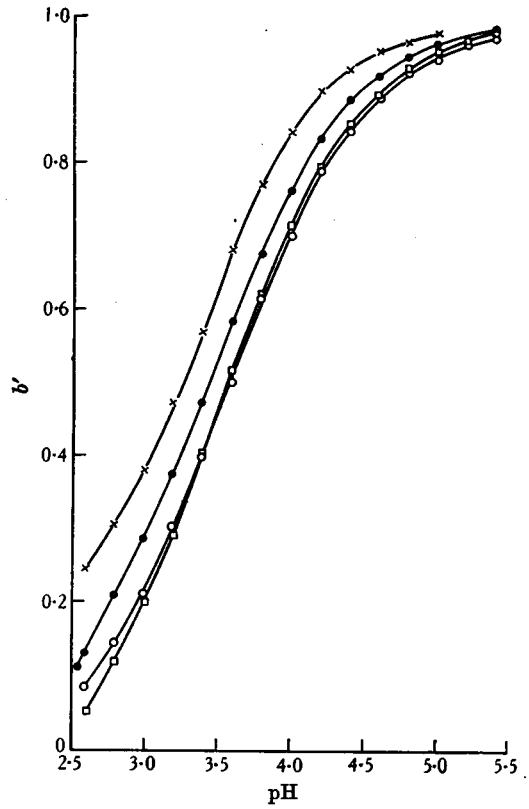

Fig. 3. Titration curves for capsular substance in N-KCl. ×——× H228B. ●——● F9. ○——○ H230B. □——□ H228A.

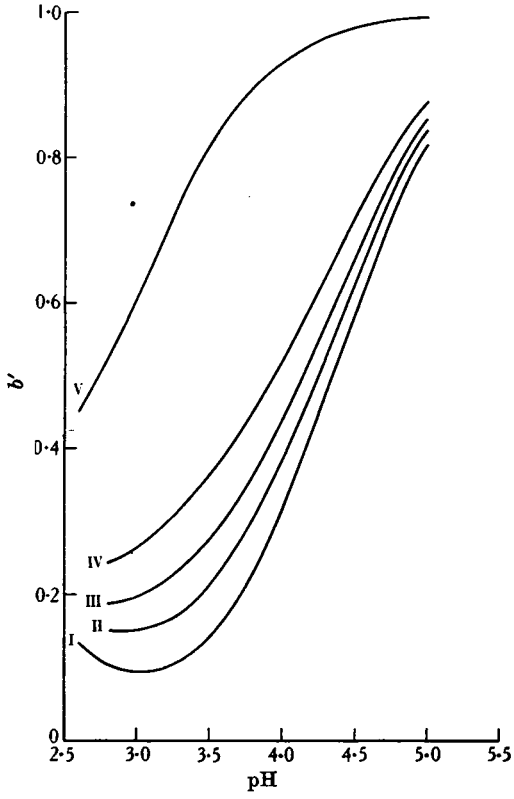

Fig. 4. Theoretical titration curves for capsular substance (H228B) in N-KCl. Curve I, $\alpha/\gamma=0$; curve II, $\alpha/\gamma=1/8$; curve III, $\alpha/\gamma=1/4$; curve IV, $\alpha/\gamma=1/2$; curve V, $\alpha/\gamma=\infty$.

by m α-peptide linkages and n γ-peptide linkages, there are $(m+n+2)$ free carboxyl groups, of which $(m+1)$ are γ-carboxyls and $(n+1)$ α-carboxyls. The dissociation constants of these two types of carboxyl group are different (in our calculations we have used the values, pK = 4·35 for γ-carboxyl and pK = 2·90 for α-carboxyl, obtained by Mr F. Call by electrometric titration of glutamyl-glutamic acid prepared by the method of Bergmann & Zervas, 1932) and it should be possible to determine the ratio of α- to γ-carboxyl groups by titration experiments. We accordingly carried out electrometric titrations with three preparations of the capsular substance with the results given in Table 4. The

Table 4. *Dissociation constants and ratio of α- to γ-carboxyl groups for the capsular substance of* B. anthracis

Preparation	Mol. wt.	No. of glutamic residues	*pG'* (in H_2O)	α/γ ratio
H228B (Na salt from HM)	53,000	350	3·84	1/8
F9 (Na salt from HM)	30,000	200	4·02	1/10
H230B (Free acid from HM)	21,000	160	4·06	1/15
H228A (Na salt from Vollum)	15,000	100	3·93	1/50

titration constants, *pG'*, determined in water, lie very close to the pK value for a γ-carboxyl group, indicating that γ-carboxyl groups preponderate in the capsular substance. Unfortunately it is not possible to evaluate the α/γ ratio by such simple titrations, for two reasons; in the first place, the salt concentration rises throughout the titration, and in the second place the mutual interference of neighbouring carboxyl groups is considerable.

We are indebted to Dr G. S. Hartley for pointing out that these difficulties could be overcome by working in N-KCl solution. Under these conditions the salt concentration clearly remains practically constant throughout the titration. In addition, neighbouring carboxyl groups will not influence one another if the KCl concentration is sufficiently high, since they will be locally neutralized by ionic atmospheres resulting from a redistribution of the K^+ and Cl^- ions; in N-KCl the ionic atmosphere radius is about 3·1 A., whereas the separation of adjacent carboxyl groups in the polypeptide is about 10 A., and the conditions for non-interference are thus fulfilled sufficiently well in N-KCl solution. In N-KCl solution the titration curves were, of course, considerably displaced; the α/γ ratios were evaluated by plotting the titration curves (pH against $b' = \alpha + h/c$; Simms, 1926; von Muralt, 1930) on tracing paper and fitting them as closely as possible to theoretical curves calculated for various α/γ ratios by means of the accurate modified Henderson equation, $pH = pK + \log b'/(1-b')$. Although a very high degree of accuracy cannot be claimed for the α/γ ratios so evaluated, there is clearly a correlation between the molecular weight and the α/γ ratio; preparations of low molecular weight contain a higher proportion of γ-carboxyl groups (and hence a lower proportion of γ-peptide linkages) than preparations of high molecular weight. This is the same conclusion as was reached by considering the amino-nitrogen results.

On the basis of this evidence we conclude that the capsular substance is built up from α- and γ-polypeptide chains of *d*-(−)-glutamic acid units. The high viscosity of solutions of the capsular substance, as compared with solutions of globular proteins of similar molecular weights, clearly indicates that the molecules are thread-like in shape; it seems probable, therefore, that the individual α- and γ-peptide chains are linked together at, or near, their ends. Accordingly we suggest a repeating structure (I) for the capsular substance (Fig. 5).

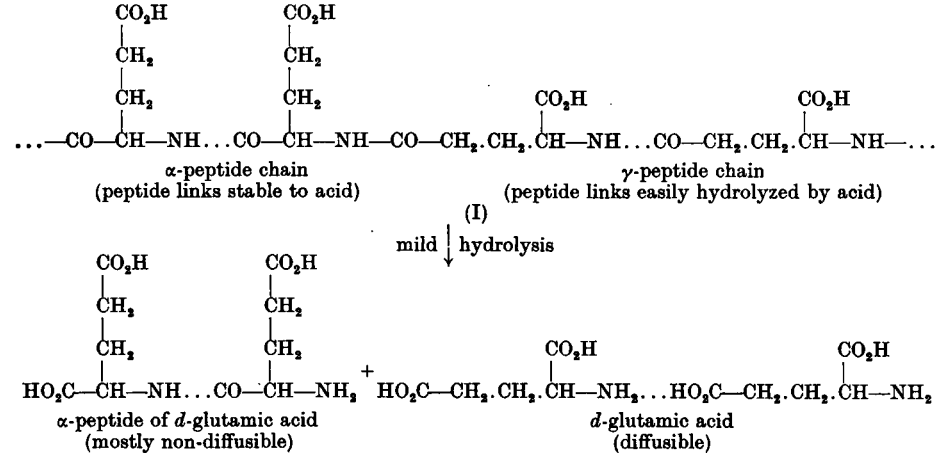

Fig. 5. The repeating structure of the capsular substance of *B. anthracis* and formulation of its hydrolytic fission.

It will be seen that the behaviour to be expected of such a substance on mild hydrolysis (involving the fission of γ-, but not α-, peptide linkages), followed by dialysis, is precisely that which was observed during the purification of the polypeptide, viz. conversion into a product of lower average molecular weight with a smaller proportion of free α-carboxyl groups.

A further quantitative experiment was carried out in which a specimen (H 230 B) of the capsular substance was dialyzed for 7 days at room temperature in 0·01-N-HCl. The experimental findings agreed with those expected for a substance of structure I (Fig. 5); about 3% of the nitrogen (representing that contained in γ-peptide linked glutamic acid residues) was found to have diffused through the membrane. 60% of this nitrogen was amino-nitrogen, indicating that the diffusible material was predominantly glutamic acid with a little peptide. The N/NH_2-N ratio of the indiffusible portion indicated a chain length of 90 glutamic acid units as compared with 160 units for the original specimen; clearly the individual α-peptide chains are themselves quite long.

It remains only to add that we have encountered no valid evidence for any chemical difference between the capsular substances produced by the two strains of *B. anthracis* used in this work.

EXPERIMENTAL

The bacterial suspensions used in this work were grown for us by Dr G. P. Gladstone by the following methods:

(i) '*Vollum*' *strain.* This strain is not normally capsulated when grown on the ordinary laboratory media, but a heavy growth of capsulated organisms can be obtained in broth cultures by adequate aeration with air containing 5–20% CO_2. For the present work the organism was grown on the acid-hydrolyzed casein—yeast extract broth ('CCY') of Gladstone & Fildes (1940).

1 l. of CCY broth was inoculated with 1 ml. of a spore suspension containing about 10^8 spores; after growing stagnant for 6 hr. this culture was added to a further litre of CCY broth in an apparatus fitted for vigorous aeration through a sintered glass disk (porosity 4). Growth was then continued at 37° for a further 16 hr. while air containing 10% CO_2 was passed through at 500 ml./min. The culture so obtained was microscopically homogeneous, consisting entirely of short chains of large, heavily capsulated bacilli; no spores were seen.

(ii) '*HM*' *strain.* This strain gives a viscous mucoid growth, showing well-defined capsules, on ordinary media (see Appendix); there is a tendency for capsule production to be lost on subculture, and repeated plating and selection of mucoid colonies was necessary. The method of growth was similar to that used by Henderson & Morgan (1938) for *Eberthella typhosa.*

Enamel photographic dishes, 10 × 14 × 1 in., each containing 500 ml. of CCY agar, were inoculated with a suspension of an 18 hr. culture by means of a glass spreader. The thick viscous growth, consisting of about 90% capsulated bacilli in chains, was scraped off with a glass plate after 18 hr. at 37° and suspended in water (25 ml. per dish).

The serological tests were also carried out by Dr G. P. Gladstone, who has supplied the following information. Two anti-sera were used, one obtained by immunizing rabbits with capsulated organisms of the Vollum strain killed with 0·001 M-$HgCl_2$, the other by immunizing rabbits with Vollum spores killed by autoclaving (it has been found that small amounts of capsular substance can be extracted from washed Vollum spores by the procedure described below). The preparation of such anti-capsular sera is difficult and erratic; full details will be published elsewhere.

The precipitation tests were carried out by mixing these sera, diluted 1 in 8, with equal volumes of serial dilutions of the capsular substance in saline. Tests were read after incubation at 37° for 1 hr. followed by keeping overnight in the refrigerator. As there was no evidence of solubility of the precipitate in excess of antiserum, readings at the end-point were considered to give an approximate indication of the amount of capsular substance present.

Isolation and purification of the capsular substance

Preliminary experiments. The experiments outlined in Table 5 were carried out with the HM strain; the organism was killed by heating to 60°. After the preliminary treatment outlined in the Table, the suspension was treated with HCl until it no longer clotted on the addition of 3 vol. of ethanol; 3 vol. of ethanol were then added and the suspension was filtered through kieselguhr or separated on the Laval centrifuge. The clear filtrate was brought to pH 8–9 with NaOH; after settling for some hours the precipitate was collected by decantation and centrifugation, washed with absolute ethanol and dried in a vacuum desiccator.

The products were all serologically active, giving precipitin reactions with anti-capsular serum down to $1 : 10^7$. All the preparations were carbohydrate free (Molisch reaction); spectroscopic examination showed the presence of large amounts of Na, Ca and Mg together with small amounts of other metals.

Clearly, simple autoclaving (process xi) was the simplest and most efficient and was adopted for the routine preparation of the capsular substance.

Recommended procedure. The following details apply to the sodium salt; other salts, or the free

Table 5. *Preliminary experiments on the isolation of the capsular substance of* B. anthracis

Procedure	Vol. of suspension used (ml.)	Yield mg.	Yield mg./ml.	Prep. no.
(i) Suspension diluted with equal vol. water. Adjusted to pH 8·5. Refluxed for 3 hr.	100	600	6·0	H208A
	1,000	6,860	6·9	R519A
	2,000	13,720	6·9	H217B
(ii) Suspension diluted with 3 vol. water. Adjusted to pH 8·5. Heated 4 hr. on boiling water-bath	200	910	4·6	H207E
(iii) Suspension diluted with equal vol. water. Adjusted to pH 9·5. Incubated at 37° for 20 hr. with 500 mg. trypsin	100	470	4·7	H216C
(iv) Suspension adjusted to pH 3–4 with HCl. Treated with 3 vol. EtOH. Shaken 18 hr.	500	3,600	7·2	R519B
(v) Suspension adjusted to pH 3–4 with HCl. 2·5 g. Na tauroglycocholate in 20 ml. water added. Kept 1 hr. at room temp.	500	2,310	4·6	R519C
(vi) Suspension brought to pH 3–4 with HCl. Kept 1 hr. at room temp.	500	3,190	6·4	H211C
(vii) Suspension diluted with equal vol. water and brought to pH 8·5. 0·1% Aerosol OT added. Kept 24 hr. at room temp.	60	220	3·7	R516A
	60	180	3·0	H209A
(viii) Suspension diluted with 3 vol. water. Brought to pH 8·5. 1% Aerosol OT added. Kept 24 hr. at 5°	50	200	4·0	R515A
(ix) Suspension brought to pH 3–4 with HCl. 0·5% Aerosol OT added. Kept 1 hr. at room temp.	500	3,710	7·4	H211B
	3,000	13,900	4·6	H215A
	1,000	5,840	5·8	H217A
(x) Suspension diluted with equal vol. water and autoclaved. Adjusted to pH 3–4. 0·5% Aerosol OT added. Kept 1 hr. at room temp.	3,000	15,000	5·0	F3
(xi) Suspension autoclaved only	2,600	21,000	8·1	F9
	3,000	32,000	10·7	F4

acid, are obtained by replacing NaCl by the appropriate chloride in the decomposition of the silver salt.

A thick, autoclaved (120° for 45 min.) suspension of the capsulated organisms is treated with sufficient 5N-HCl to prevent separation of a bulky flocculent precipitate on dilution with alcohol. Ethanol (3 vol.) is then added and bacterial bodies, etc. are removed by centrifugation (Laval) or filtration. Sufficient ethanol is added to the clear filtrate to make up for losses by evaporation during the clarification process and 10% NaOH is added to bring the pH to 9. The precipitated crude polypeptide is collected by decantation and centrifugation. After drying in a vacuum desiccator the crude product is dissolved, with gentle warming, in sufficient water to give a not too viscous solution. After adjusting to pH 7·4 an excess of silver nitrate solution is added (the yellow precipitate flocculates and settles readily when a sufficient excess has been added). The silver salt is filtered off, washed well with water and suspended in an excess of NaCl solution; the mixture is shaken for 30 min. and kept in the refrigerator overnight. AgCl is removed by filtration (finally through kieselguhr) and the filtrate is brought to pH 9 and dialyzed against frequent changes of distilled water, brought to pH 9 with NaOH, until free from chloride. Dialysis is continued against distilled water until the contents of the dialysis bag reach pH 7·5; the dialyzed solution is then evaporated, first under reduced pressure at room temperature and finally from the frozen state over P_2O_5.

The sodium salt of the capsular substances is so obtained as a colourless or pale yellow crisp glass permeated with air bubbles and of reasonably uniform composition; if the final freeze-drying is omitted, the product is a horny mass the moisture content of which is very dependent on the thickness of the film.

Composition of the capsular substance

Preparation of d-*glutamic acid hydrochloride.* Organisms of the HM strain, from growth on 60 agar trays, are suspended in 10 l. of water, autoclaved and separated on the Laval centrifuge. The fluid is adjusted to pH 8 and treated with an excess of 10% copper sulphate solution (*c.* 500 ml.). After allowing to settle overnight the copper salt is filtered off, washed thoroughly with water and refluxed with 6N-HCl (600 ml.) for 2½ hr. After the addition of 200 g. of ice, H_2S is passed in for 10 min. The precipitated CuS is filtered off and the filtrate is concentrated under reduced pressure to about 100 ml. After keeping overnight in the refrigerator, the crude *d*-glutamic acid hydrochloride (20 g.) is collected by filtration, washed with a little cold conc. HCl and dried in a vacuum desiccator; a small second crop can be obtained from the mother liquor by concentration and saturation with HCl. Recrystallization, by saturation of the aqueous solution with HCl, gives, without much

loss, pure *d*-(−)-glutamic acid hydrochloride, m.p. 206°, $[\alpha]_D^{11°}$ −24·2° (*c* = 0·83 in N-HCl) (lit.: m.p. 201°; $[\alpha]_D$ −24·7°).

Chromatographic determination of glutamic acid as pyrrolidone-carboxylic acid. The following experimental procedure has been found satisfactory:

25 mg. of a mixture of amino-acid hydrochlorides (e.g. an evaporated protein hydrolysate) is treated with an amount of 0·1 N-NaOH exactly equivalent to the combined HCl present; 4 ml. of water and 2·5 ml. of acetic acid are added to the solution, bringing it to pH 2. The solution is heated in a sealed tube at 130° for 4 hr. and then evaporated to dryness at room temperature in a vacuum desiccator over KOH. The product is transferred by means of 17% butanol-chloroform to a Synge column made up from 3 g. of suitable silica gel, 1·5 ml. of 0·025% indicator *R*-NH_4 (Liddell & Rydon, 1944) and 3% butanol-chloroform. The blue band of pyrrolidone-carboxylic acid (*R* = *c.* 0·3) is eluted from the column with 17% butanol-chloroform; the solvent is evaporated from the eluate *in vacuo* and the residue titrated with 0·01 N-baryta, using bromthymol blue as indicator.

The details of a typical experiment with *l*-(+)-glutamic acid hydrochloride were as follows:

Material used: 25·0 mg. (0·1362 mmol.).

Pyrrolidone-carboxylic acid: Titre = 13·0 ml. of 0·010 N-baryta.

Therefore recovery = 0·1300 mmol. = 95·4%.

Residue after transference to column: Titre = 0·50 ml. of 0·010 N-baryta.

Therefore loss = 0·0050 mmol. = 3·7%.

In a duplicate experiment the recovery of pyrrolidone-carboxylic acid was 95·8% and the loss in the residue 3·0%.

Hydrolysis of capsular substance from the HM strain. 212·0 mg. of the sodium salt F 9 were heated in a sealed tube at 100° for 18 hr. with 6 N-HCl (24 ml.) and acetic acid (6·4 ml.). The hydrolysate was evaporated to dryness *in vacuo* and kept for several days in a vacuum desiccator over KOH and silica gel. A stock solution was prepared by dissolving the residue in water (total vol. = 10 ml.).

Analysis of this stock solution gave the following results:

Total nitrogen	1·74 mg./ml.
Amino-nitrogen	1·72 mg./ml.
Amide-nitrogen	Nil
Chloride ion	0·250 m-equiv./ml.

1 ml. gave no precipitate with 1 ml. of a saturated solution of Reinecke's salt or with 1 ml. of a 15% solution of phosphotungstic acid in 5% H_2SO_4.

1 ml. was treated with 1·25 ml. of 0·1 N-NaOH (equivalent to the combined HCl, the remainder of the chloride ion being present as NaCl); 3 ml. of water and 2·5 ml. of glacial acetic acid. After heating at 130° for 4 hr., the solution was evaporated and the pyrrolidone-carboxylic acid transferred to a silica-gel column; the single blue band had *R* = 0·30. The evaporated eluate required 11·70 ml. of 0·01 N-baryta for neutralization. This corresponds to 1·64 mg. of nitrogen (uncorrected) or 1·71 mg. (= 98% of the total nitrogen) after correcting for the losses observed with authentic glutamic acid. In a repeat experiment the titre was 12·00 ml. of 0·01 N-baryta, i.e. 1·68 mg. N (uncorrected) or 1·75 mg. N (corrected), corresponding to 101% of the total nitrogen. The residue left after transferring the pyrrolidone-carboxylic acid to the column was dried, dissolved in 3 ml. of boiling water and acetylated by the addition of 1 ml. of acetic anhydride to the boiling solution in small quantities with shaking. The evaporated product was transferred to a silica-gel column with 17% butanol-chloroform; two small bands were observed (*R* = 0·29, corresponding to pyrrolidone-carboxylic acid, and *R* = 0·12, corresponding to acetylglutamic acid) but disappeared before leaving the column; no bands corresponding to other amino-acids could be detected. Control experiments showed that small amounts of acetyl-aspartic acid or acetylglycine would probably have been detected at this stage.

Hydrolysis of capsular substance from the Vollum strain. 208·0 mg. of the sodium salt H 228 A were heated in a sealed tube at 100° for 18 hr. with 6 N-HCl (24 ml.) and acetic acid (6·4 ml.). The hydrolysate was treated as described above and a stock solution (10 ml.) made up. This gave no precipitate with a saturated solution of Reinecke's salt or with 15% phosphotungstic acid in 5% sulphuric acid.

Analysis of the stock solution gave the following results:

Total nitrogen	1·76 mg./ml.
Amino-nitrogen	1·71 mg./ml.
Amide-nitrogen	0·032 mg./ml.
Chloride ion	0·244 m-equiv./ml.

1 ml. of stock solution was subjected to the process for conversion into pyrrolidone-carboxylic acid. The pyrrolidone-carboxylic acid band had *R* = 0·30; titration (required 11·65 ml. of 0·010 N-baryta) showed that the band corresponded to 1·63 mg. (uncorrected) or 1·71 (corrected) of nitrogen; the latter figure accounts for 99% of the non-amide-nitrogen. Acetylation and partition chromatography of the residue gave only one very small band (*R* = 0·12, corresponding to acetylglutamic acid) which disappeared before it left the column.

The following results were obtained in a repeat experiment with another sample of H 228 A; the hydrolysate was made up to 10 ml.:

Total nitrogen	1·025 mg./ml.
Amino-nitrogen	0·99 mg./ml.
Amide-nitrogen	0·024 mg./ml.
Glutamic acid-nitrogen	1·00 mg./ml. = 100% of non-amide-nitrogen

Structure of the capsular substance

Hydrolysis in acid solution. A solution of crude capsular material (C154B; *c.* 10 mg./ml.) was brought to pH 2 with HCl and kept at 25°, samples being removed from time to time for determination of amino-nitrogen. The following results, expressed as % NH_2-N on the original dry weight, were obtained:

Time (days):	0	1	2	7	14
% NH_2-N:	0·055	0·087	0·117	0·167	0·398

Electrometric titrations. The instrument used was a Cambridge pH meter with glass and calomel electrodes.

(i) *Titrations in water.* The substance was dissolved in a known volume of water, containing a known excess of standard acid, and back titrated with 0·1045N-baryta; readings on the pH meter were taken at intervals of 0·10 ml. The solutions were stirred by a current of nitrogen. The observations were plotted and b' (Simms, 1926) was calculated at pH intervals of 0·20; the titration constants (pG' = pH when $b' = 0{\cdot}50$) were read off from the curves relating pH to b' which are shown in Fig. 2. The details of a typical experiment are given in Table 6.

(ii) *Titrations in* N-KCl. The technique was similar to that used for the experiments in water, but the solutions were initially made up to be N in KCl, and an equal volume of 2N-KCl was added after each addition of baryta and before taking the pH reading. The details of a typical experiment are given in Table 7 and the pH-b' curves in Fig. 3.

Theoretical titration curves for the case of H228B are shown in Fig. 4. Similar sets of curves were constructed for H230B and H228A but are not reproduced.

Dialysis in acid solution. H230B (116·2 mg.) was dissolved in water (10 ml.) and dialyzed for 7 days at room temperature against 220 ml. of 0·01N-HCl, precautions being taken to prevent ammonia, etc. entering the solutions from the atmosphere. The solutions were then evaporated to dryness, first *in vacuo* at room temperature and finally from the frozen state over P_2O_5. Analysis gave the following results:

	Total N (mg.)	NH_2-N (mg.)	(Total N/NH_2-N) = mean chain length
Dialysate	0·364	0·206	1·8
Residue	11·4	0·126	90

No pyrrolidone-carboxylic acid could be detected in the dialysate by the adsorption-chromatographic method.

Table 6. *Electrometric titration of capsular substance in water*

H228B. 88·7 mg. in 6·00 ml. 0·1003N-HCl and 14·00 ml. water. Na content of H228B = 13·34%. Hence HCl required = 0·5145 m-equiv. Therefore excess HCl added = 0·0873 m-equiv. Equiv. (from titration to pH 7) = 172·3. Temp. = 20°.

pH	Baryta added (ml.)	b	a	h	c	b'
2·35	0·00	0·000000	0·004365	0·00447	0·02573	0·004
2·40	0·16	0·000830	0·004332	0·00398	0·02553	0·031
2·60	0·62	0·003142	0·004234	0·00251	0·02495	0·057
2·80	1·00	0·004976	0·004158	0·00158	0·02450	0·098
3·00	1·38	0·006745	0·004083	0·00100	0·02406	0·153
3·20	1·72	0·008273	0·004020	0·00063	0·02369	0·206
3·40	2·14	0·01010	0·003943	0·00040	0·02324	0·282
3·60	2·60	0·01202	0·003863	0·00025	0·02277	0·369
3·80	3·16	0·01426	0·003770	0·00016	0·02222	0·479
4·00	3·70	0·01631	0·003684	0·00010	0·02171	0·586
4·20	4·20	0·01813	0·003608	0·00006	0·02126	0·699
4·40	4·70	0·01988	0·003534	0·00004	0·02083	0·787
4·60	5·02	0·02097	0·003489	0·000025	0·02057	0·851
4·80	5·26	0·02176	0·003456	0·00002	0·02037	0·900
5·00	5·44	0·02235	0·003432	0·00001	0·02022	0·936
5·20	5·58	0·02279	0·003413	0·00001	0·02011	0·964
5·40	5·70	0·02318	0·003396	0·00000	0·02002	0·989

The symbols at the heads of columns 3–7 have the following significance:

b = concentration (equiv./l.) of added base (baryta),
a = „ „ of added acid (HCl),
h = „ „ of hydrogen ion,
c = „ „ of polypeptide,

$$b' = \frac{b - a + h}{c}.$$

Table 7. *Electrometric titration of capsular substance in* N-KCl

H228B. 85·5 mg. in 6·00 ml. 0·1003 N-HCl, 4·00 ml. water and 10·00 ml. 2 N-KCl. Na content of H228B = 13·34%. Hence HCl required = 0·4961 m-equiv. Therefore excess HCl added = 0·1057 m-equiv. Equiv. = 172·3. Temp. = 17·5°.

pH	Baryta added (ml.)	*b*	*a*	*h*	*c*	*b'*
1·95	0·00	0·000000	0·005285	0·01122	0·02481	0·239
2·00	0·16	0·000823	0·005201	0·01000	0·02442	0·230
2·20	0·72	0·003510	0·004929	0·00631	0·02314	0·211
2·40	1·22	0·005682	0·004710	0·00398	0·02211	0·224
2·60	1·62	0·007285	0·004547	0·00251	0·02135	0·246
2·80	2·10	0·009067	0·004367	0·001585	0·02050	0·307
3·00	2·58	0·01072	0·004201	0·00100	0·01972	0·381
3·20	3·10	0·01237	0·004033	0·00063	0·01893	0·474
3·40	3·62	0·01389	0·003881	0·00040	0·01822	0·571
3·60	4·18	0·01540	0·003727	0·00025	0·01750	0·682
3·80	4·62	0·01651	0·003614	0·00016	0·01696	0·769
4·00	4·98	0·01737	0·003527	0·00010	0·01656	0·842
4·20	5·26	0·01801	0·003462	0·00006	0·01626	0·899
4·40	5·40	0·01832	0·003431	0·00004	0·01611	0·927
4·60	5·54	0·01862	0·003400	0·000025	0·01596	0·955
4·80	5·60	0·01875	0·003387	0·00002	0·01590	0·967
5·00	5·67	0·01888	0·003375	0·00001	0·01584	0·979

Symbols have same significance as in Table 6.

SUMMARY

1. Methods are described for the isolation and purification, without undue degradation, of the capsular substance from two strains of *B. anthracis*.

2. There is no evidence for any chemical differences between the capsular substances obtained from these two strains.

3. The capsular substance is shown to be made up solely from *d*-(−)-glutamic acid residues; the molecular weight of the native material is greater than 50,000 and it is thus of the same order of size as many proteins.

4. Structurally, the capsular substance is a long-chain molecule made up of α-peptide chains of 50–100 *d*-glutamic acid residues joined together by γ-peptide chains of *d*-glutamic acid residues (see p. 302).

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Appendix. Note on the Selection of Non-sporing and Mucoid Variants from a Strain of *B. anthracis* and on the Extraction from the Mucoid Form of the Capsular Substance

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In the course of some work on *B. anthracis* the problem arose of separating from a classic culture of that organism non-sporing races and races similar to the strains referred to by Tomcsik & Szongott (1933) and Ivánovics & Erdös (1937), showing free development of capsules under ordinary conditions of cultivation *in vitro*. Tentatively I applied to the strain 'Vollum' the method of treatment with activated specific serum which I had employed with success for the isolation of rough and other variants from vibrio cultures. For this enterprise there was available in the first place a sample of a veterinary anti-anthrax serum from Budapest, and later a sample of Sclavo's serum prepared by the Tuscan Serum and Vaccine Institute, Sienna. With slight variations the mixtures prepared were as follows:

	Tubes 1 (*a*, *b* and *c*) (ml.)	Tubes 2 (*a*, *b* and *c*) (ml.)	Tubes 3 (*a*, *b* and *c*) (ml.)	Tubes 4 (*a*, *b* and *c*) (ml.)
Anti-anthrax serum diluted 1 : 50	1	0·5	0·2	0·1
Saline	—	0·5	0·8	0·9
Fresh guinea-pig serum (complement) diluted (with nutrient broth) in series: (*a*) 1 : 5, (*b*) 1 : 10, (*c*) 1 : 15	0·5	0·5	0·5	0·5

To the 1·5 ml. contents of each of the twelve experimental tubes and of controls lacking either complement or specific serum was added a drop of a faintly turbid suspension of a 12 hr. culture of the 'Vollum' strain of *B. anthracis*.

After 12 and 18 hours' incubation at 37° the mixtures were plated out on nutrient agar. In these platings, together with colonies of the classic type there appeared in many instances a number of the circular, raised, opaque, moistly caseous and yellowish colonies of the type which—though 'roughness' is not yet known to occur in this group of bacilli—has sometimes been called 'smooth'. These latter organisms proved to be asporogenous and, though those isolated by the agency of the Budapest serum eventually resumed sporulation in subculture, many of those obtained by use of the Sclavo serum were fixed in their peculiarity and have long been propagated on solid and in liquid media without forming elements surviving 20 min. heating at 60°.

In platings of cultures treated with the Budapest serum there appeared also colonies of mucoid aspect and consistence formed of magnificently capsulated bacilli. The condition was transmissible in subculture but the cultures were apt to contain spores. To obtain races both capsulate and absolute in asporogenicity the attempt was made to select by means of the Budapest serum mucoid races from the non-sporing Sclavo-won Hampstead (or H) variants, which, like the 'Vollum' parent showed in ordinary culture occasional capsulate units. The trial was entirely successful and there were obtained the Hampstead Mucoid (or HM) series of variant races (Plate 4) which have since been maintained in full character by intermittent selection of colonies of mucoid habit.

It may be noted that at first isolation the H variants tested showed virulence for guinea-pigs and mice not markedly less than that of the parent culture while the virulence of the HM variants was from the outset of a much lower order.

Being in total ignorance of the serological factors involved in the somewhat differing action of the two antisera I am unable to point the rationale of the events outlined. It is, however, to be suspected that the variants presented themselves rather through the selective than the stimulant action of the treatment to which the parent culture was subjected. Over the range of the experiments made no special relation between the serum dose employed and the appearance of the variants was observed.

Extraction of capsular material

Extraction of the capsular material, offered in abundance by the HM cultures, was undertaken; it was quickly found that this could be achieved by ethanol containing mineral acid—the solvent previously employed for the extraction of ethanol-soluble proteins from Gram-negative bacilli (White, 1932, 1934, 1936).

The organism was grown in mass in enamelled trays, 24 trays affording 20 sq.ft. of nutrient agar surface. The slimy growth, collected after 24–48 hr. incubation at 37° and killed and dispersed in water by steaming, was diluted till it could be refluxed on a sand bath, which process, possibly unnecessary and even undesirable, was maintained for some hours. A pilot experiment having been made on a small sample of the material, the bulk was mixed with 3 vol. of ethanol and the requisite amount of hydrochloric

Plate 4. Micro-photograph of a young culture of an HM variant of *B. anthracis* ('Vollum') positively stained with methylene blue and negatively stained with congo red. The capsules are shown in relief. In some rods capsulation has not yet developed or is in process of development. Photograph by Mr R. McV. Weston, F.R.M.S., F.R.P.S. (× 1500).

Appendix. Note on the Selection of Non-sporing and Mucoid Variants from a Strain of *B. anthracis* and on the Extraction from the Mucoid Form of the Capsular Substance.

By P. Bruce White

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for molecular weight at about 9000. *read* molecular weight at about 22,400.

acid was slowly added with stirring to disperse, by dissolution of the capsular substance, the stringy coagulum first formed, leaving a granular precipitate. The ethanolic supernatant, thoroughly filtered, was treated with NaOH solution until with neutralization maximum precipitation had occurred. After re-solution in acid 75% (v/v) ethanol, re-filtration and re-precipitation with NaOH, this precipitate, dissolved in a small volume of water, was subjected to dialysis against N/50 HCl and finally against distilled water—a precipitate forming during dialysis being removed by centrifugation. On evaporation the dialyzed solution became viscid as dryness was approached and the capsular material was eventually left as a tough vitreous layer on the floor of the containing dish from which it was parted with difficulty, emitting at separation an almost metallic 'cry'.

This material showed the general features of the products of Tomcsik and Ivánovics and their collaborators and, though reacting in high dilution with the only anti-P (anti-capsular substance) serum prepared against the whole organism, failed itself to engender a formation of antibodies in any of several rabbits injected with its solutions.

There was observed its ability to form precipitates with methylene blue and atebrin and Dr Harold King noted a similar reaction with diamidines. My colleague, Mrs R. V. Pitt Rivers, who further purified and freeze-dried a sample of the substance, from free amino-N determinations assessed the molecular weight at about 9000.

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The Manometric Determination of the Activity of Carbonic Anhydrase under Varied Conditions

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The activity of carbonic anhydrase was first measured by Meldrum & Roughton (1932) with the boat-manometric method which Brinkman, Margaria & Roughton (1933) had developed for study of the kinetics of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ in absence of enzyme. In this method a buffer solution is shaken violently with bicarbonate solution (or with a CO_2-containing gas phase) in a boat-shaped vessel which is flexibly connected to a U-tube manometer. The rate of CO_2 output (or uptake) is followed by reading the pressure at various times after the shaking begins. The magnification of the rate, when carbonic anhydrase is added to the buffer, is taken as a measure of the activity of the enzyme. Variations and improvements in the manometric method have since been made by Stadie & O'Brien (1933), Van Goor (1934), Roughton & Booth (1938), Kiese & Hastings (1940), Kiese (1941), and Leiner (1941).

In all such work diffusion between the gas and liquid phases tends to become a limiting factor as the reaction velocity of the chemical process rises. The influence of diffusion can, however, be allowed for quantitatively by the theoretical and experimental methods recently worked out by Roughton (1941) on the basis of the stationary film theory of gas-liquid interchange.

In another set of methods the time is measured for the reaction to bring about a certain change in pH in absence and in presence of the enzyme. Stadie & O'Brien (1933) followed the pH electrometrically, but most authors have preferred the much more simple methods with indicators (Brinkman, 1933; Van Goor, 1934; Philpot & Philpot, 1936). These methods are, however, in general much less powerful and definite than manometry as regards the range of conditions under which the activity of the enzyme can be investigated.

All the methods hitherto used are fairly adequate for determining the number of enzyme units present in a given preparation and have accordingly been much used in

(i) The study of the distribution of the enzyme in the blood, body fluids and tissues of a wide range of organisms with a view to unravelling its role in CO_2 transport, gastric secretion and other physiological processes.

(ii) The development of methods of purifying the enzyme, culminating recently in its crystallization by Scott & Fisher (1942).