Physicochemical Examination of Polyglutamic Acid from *Bacillus anthracis* Grown *in vivo*

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The two specimens of polyglutamic acid investigated were isolated by our colleagues H. T. Zwartouw and H. Smith from guinea pigs infected with *Bacillus anthracis*, as described in a previous paper (Zwartouw & Smith, 1956). These specimens were: (*a*) extracellular material obtained from the plasma exudate of the infected animals; (*b*) capsular material from the organisms separated from the blood of the animals.

Both specimens were obtained as sodium salts, containing about 14·8 % of sodium, and were dialysed salt-free and freeze-dried. They have been examined in the electrophoresis apparatus and the ultracentrifuge for homogeneity, and sedimentation and diffusion coefficients have been determined over a range of concentration to estimate their molecular weights. The special difficulties arising from the highly ionic nature of polyglutamic acid, a typical polyelectrolyte, are briefly discussed.

METHODS

Electrophoresis. The experiments were carried out at 0° in the 10 ml. U-tube of the Tiselius electrophoresis apparatus, with the diagonal-edge schlieren optical system for observation. Solutions were made up to about 1% from the freeze-dried sample and were dialysed for 2 days against the buffer used.

Ultracentrifuge. Observations were made in the Spinco electrically driven ultracentrifuge with the diagonal-bar schlieren optical system. Apart from those examined by electrophoresis, solutions were made up directly in the buffer by weighing the freeze-dried sample with suitable precautions against absorption of atmospheric moisture. The synthetic boundary cell of Pickels, Harrington &

Schachman (1952) proved a decided advantage in making measurements at low concentrations and in strong salt solutions. The rotor speed was 59 780 rev./min. in all experiments.

Diffusion. The measurements were carried out in a Gouy interferometric diffusion apparatus similar to that of Gosting, Hanson, Kegeles & Morris (1949) in design and with a cell based largely on that of Ogston (1949), of Coulson, Cox, Ogston & Philpot (1948) and of Creeth (1952). The cell was immersed in a well-stirred thermostat at 25±0·01°. Special attention was paid to thorough dialysis of the solutions by continuous slow rotation of the cellophan bags (2 days at 3°) and to preventing evaporation losses at all stages. These precautions become especially important with strong salt solutions as in the present work.

RESULTS

Extracellular polyglutamic acid

Electrophoresis. Fig. 1 shows the pattern obtained for an approx. 1 % solution in pH 8 phosphate (I 0·1, 0·0022M-KH_2PO_4, 0·0326M-Na_2HPO_4)–NaCl (I 0·1) buffer in the long-limb tube after prolonged electrophoresis. A single component only is observed. The sharp-ascending and diffuse-descending boundaries, as also the pronounced almost stationary δ and ϵ boundaries in these relative concentrations of material and buffer, are indicative of the highly ionic nature of the material. The asymmetry of the cathode boundary, due possibly to polydispersity of the material, makes precise measurement of mobility difficult. The value calculated from the rate of movement to the anode of the centroid of the cathode boundary is $1·50 \times 10^{-4}$ cm.^{2}/sec./v.
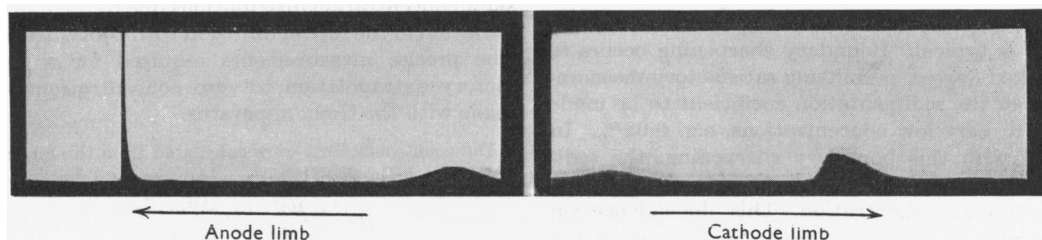

Anode limb   Cathode limb

Fig. 1. Electrophoresis patterns of extracellular sodium polyglutamate in pH 8 phosphate (I 0·1)–NaCl (I 0·1) buffer. The arrows indicate the direction of migration towards the anode. Exposure after 6 hr. 7 min.; current, 9·0 mA.
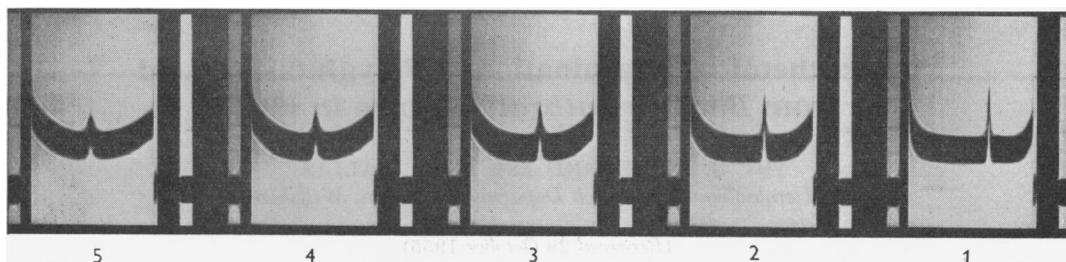
Fig. 2. Sedimentation pattern of extracellular sodium polyglutamate in pH 8 phosphate (I 0·1)–KCl (I 1·0) buffer. Concentration 0·098 g./100 ml. Speed, 59 780 rev./min. Interval between exposures, 8 min. First exposure taken 1 min. after reaching full speed, with the synthetic-boundary cell.

A similar picture was obtained with acetate (I 0·1, 0·15M acetic acid, 0·1M sodium acetate)–NaCl (I 0·1) buffer, pH 4·4, but the δ and ε buffer gradients were less prominent and the mobility of the cathode boundary, though still anodic, was somewhat reduced.

Sedimentation. When examined in the ultracentrifuge, both the solutions used for the electrophoretic analysis showed a single, very sharp, sedimenting boundary at both pH values. The single boundary, in sedimentation as well as electrophoresis, is in harmony with our concept of the material as having, if not an entirely monodisperse character, then a distribution of molecular weights about a single mean.

In the quantitative study of the sedimentation for the purpose of assessing the molecular weight, a stronger salt solution was considered desirable to suppress primary charge effects. This, together with the further requirement of avoiding secondary charge effects, led to the choice of M-KCl. Phosphate buffer (pH 8, I 0·1, as described above) was incorporated to stabilize the pH (measured pH 7·5). A buffer of this concentration develops an appreciable refraction gradient in the ultracentrifuge cell and these studies have been facilitated by the use of the synthetic-boundary cell, by which means the boundary is formed near the cell centre during the running up to speed. The procedure has the added advantage that the boundary movement can be measured from the moment full speed is attained.

The diagonal-bar schlieren pattern shown in Fig. 2 is typical. Boundary sharpening occurs to a marked degree, permitting satisfactory measurements of the sedimentation coefficient to be made down to very low concentrations, e.g. 0·02 %. In accord with this boundary sharpening, the sedimentation rate is found to increase considerably with falling concentration. This dependence on concentration, combined with the dilution in the cell during sedimentation leads, during a long run, to an increase in S during the run which, if the
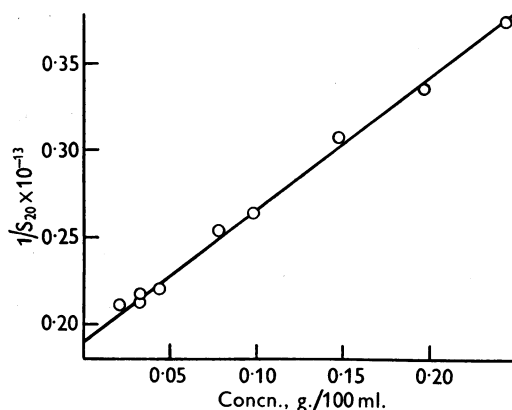

Fig. 3. Sedimentation coefficients of extracellular sodium polyglutamate in M-KCl–phosphate buffer.

boundary shift is large, necessitates the use of the $(x/x_0)^2$ correction for dilution during sedimentation. Fig. 3 shows the reciprocals of the sedimentation coefficients, uncorrected for the viscosity and buoyancy of the buffer, at 20° plotted against concentration. All the points lie, within experimental error, on a straight line which extrapolates to zero concentration to give $S_{20} = 5·26 \times 10^{-13}$.

Diffusion. Preliminary diffusion studies in the Tiselius apparatus with 0·5 % solutions showed, as expected from the behaviour in the ultracentrifuge, skew concentration distributions due to the marked variation in the rate of diffusion with concentration. The precise measurements required for a satisfactory extrapolation to zero concentration were made with the Gouy apparatus.

Diffusion coefficients were calculated from the formulae of Kegeles & Gosting (1947):

$$D = \frac{(j_m \lambda b)^2}{4\pi C_t^2 t} = \frac{(j_m \lambda b)^2 (e^{-z_j^2})^2}{4\pi Y_{j_0}^2 t}, \qquad (1)$$

where j_m is given by $j_m = a(n_s - n_0)/\lambda$, and where a is the width of the liquid column in the direction of the light

beam; b is the optical distance from the centre of the diffusion cell to the photographic plate; λ is the wavelength of light *in vacuo* used for observation; t is the time in seconds between the formation of the infinitely sharp boundary and the time of observation; Y_{j_o} is the distance of the outermost minimum from the normal slit image position; n_s and n_o are the refractive indices of the solution and solvent referred to vacuum.

The quantity, $e^{-z_j^2}$ was obtained from tables of $e^{-z_j^2}$ against a function $f(z_j)$, where

$$f(z_j) = \frac{2}{\sqrt{\pi}} \int_0^{z_j} e^{-\beta^2} d\beta - \frac{2}{\sqrt{\pi}} z_j e^{-z_j^2}$$

and was derived from calculations of the interference conditions by use of the Airy integral (see Gosting & Morris, 1949). z_j represents the optical 'reduced height' in the cell corresponding to a given maximum or minimum at the plate. The quantity C_t, defined by $Y_j = C_t e^{-z_j^2}$, represents the maximum downwards displacement of light at the photographic plate if the light followed geometrical optics; for an indeal diffusion C_t should be constant for each fringe of a pattern at a given time.

In equation (1) it was found that values calculated from the first part of the relation (C_t method) were about 1 % lower than those calculated from the second (Y_{j_o} method).

Besides the evaluation of j_m from a summation of the integral number of fringes plus the fractional part (derived from the displacement of Rayleigh fringes whilst the boundary was sharpening), approximate values of j_m were derived from the Gouy patterns at different stages of each diffusion experiment. Denoting these latter values as j'_m, it was invariably found that a decrease in j'_m took place as diffusion proceeded, though under comparable conditions, in experiments with bovine-plasma albumin, j'_m was constant and in good agreement with j_m. The rate of fall of j'_m was greatest at the beginning of diffusion; therefore, for each experiment a single value of j'_m was used for insertion in the equations, which was the value corresponding to the time (towards the end of the diffusion experiment) when the rate of change of j'_m with time was at a minimum. Fig. 4 shows plots of C'_t against $Z_j^{\frac{2}{3}}$, where $C'_t = Y_j/e^{-z_j^2}$ (see Akely & Gosting, 1953) for a selection of exposures taken after different periods of diffusion at one concentration. The relationship is very closely linear. If, however, the value j_m had been used in place of j'_m the deviations from linearity would have taken place at lower values of $Z_j^{\frac{2}{3}}$. No further attempts have been made to analyse the deviations of C'_t from its value at $Z_j^{\frac{2}{3}} = 0$.

The variation of the diffusion coefficient, $D_{20,w}$, with concentration of solute is shown in Fig. 5 (lower line). The results were calculated by using the first part of equation (1) and are plotted against j'_m as a measure of solute concentration.
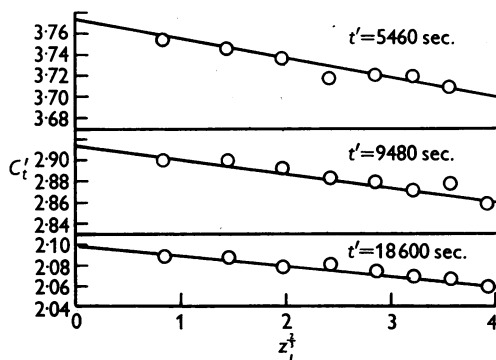

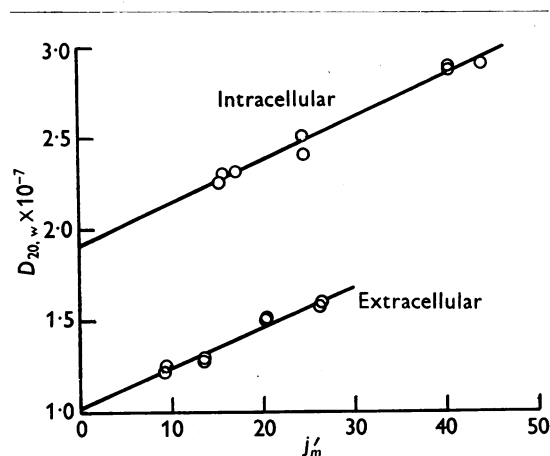Fig. 4. Extracellular sodium polyglutamate. Diffusion (see text).

Fig. 5. Diffusion coefficients of sodium polyglutamate in M-KCl–phosphate buffer.

The straight-line extrapolation has been derived by the method of least squares. Assuming such a linear relation to be valid, the value of $D_{20,w}$ at zero concentration is 1.02×10^{-7} or 1.01×10^{-7} if uncorrected for the viscosity of the buffer. We have reason to suppose from light-scattering measurements on a sample of polyglutamic acid prepared from *B. anthracis* grown *in vitro* that the second virial coefficient Γ_2 is of such magnitude at I 1·1 that a linear extrapolation of diffusion coefficients to zero concentration leads to very little error in the $D_{20,w}$ ($c \to 0$) value (see, for example, Mandelkern & Flory, 1951).

Molecular weight. In the absence of precise information on the extent and nature of any solvation, we have used the simple Svedberg equation for calculating the molecular weight:

$$M = \frac{RTS}{D(1 - V\rho)},$$

substituting the limiting value for S (uncorrected for the buoyancy and viscosity of the buffer) and D (uncorrected for viscosity) and the pyknometric (anhydrous) value for V, ρ being the density of the buffer. On theoretical grounds the sedimenting species may be either the polyglutamate ion or the salt, depending on the degree of ion-pair formation. In the case of neutralized polymethacrylic acid, Huizenga, Grieger & Wall (1950) have shown ion-pair formation to the extent of 60 % even in the absence of added electrolyte. We assume therefore that in the present case ion-pair formation approaches 100 %, and that since the sedimentation and diffusion coefficients have been determined in M-KCl buffer and extrapolated to zero concentration we are dealing effectively with measurements on the potassium rather than the sodium salt of polyglutamic acid. The partial specific volume of the sodium salt was determined pyknometrically in the M-NaCl–phosphate (I 0·1) buffer as 0·510 for a 1·5 % solution and 0·504 for a 0·78 % solution. The transfer from Na to K at molar concentration is accompanied by an increase in molar volume of about 10·3 [see Harned & Owen (1950), p. 253]. With a mean value of 0·507 for the partial specific volume of the sodium salt this leads to 0·518 for the potassium salt in M-KCl. The buffered KCl used in this work had a measured density of 1·048 at 20°. From these data a figure of 278 000 is obtained for the molecular weight of the potassium salt, corresponding to a value of 216 000 for the free acid, and this figure should represent the true anhydrous molecular weight in the absence of solvation, or in the presence of solvation provided that it is not selective. The results for the potassium salt lead to the high value of 5·4 for the frictional ratio f/f_0.

Capsular polyglutamic acid

Electrophoresis. The sample was examined at pH 8·0 and pH 4·4 under conditions similar to those already described for the extracellular material, and practically identical behaviour was observed, namely, a single migrating component with a high anodic mobility at both pH values and pronounced buffer boundaries.

Sedimentation. In the ultracentrifuge, the material behaved in a similar manner to the extracellular sample, but showed a less pronounced variation in sedimentation rate with change of concentration. At comparable concentration the sedimentation rate was appreciably slower and the boundary remained less sharp and it was not possible on this account to carry the measurements down to such low concentrations. The $1/S$–c plots again lie closely on a straight line (Fig. 6), which extrapolates to $S_{20}(c \to 0) = 3·45 \times 10^{-13}$ in the M-KCl–phosphate (I 0·1 as above) buffer used.
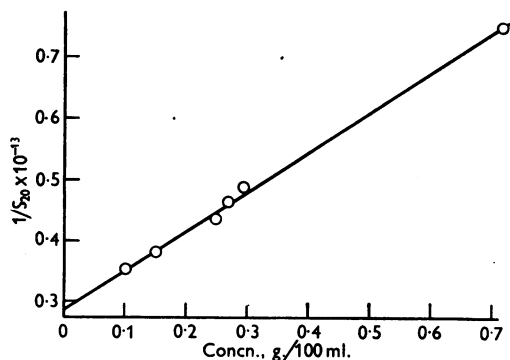

Fig. 6. Sedimentation coefficients of intracellular sodium polyglutamate in M-KCl–phosphate buffer.

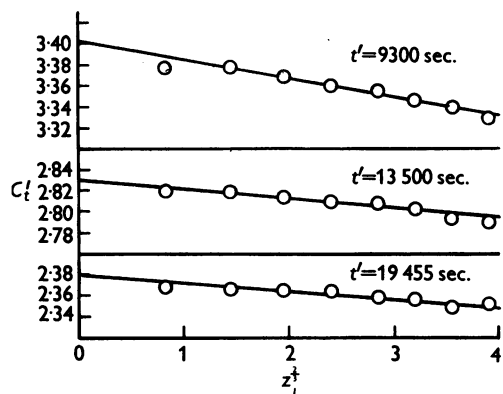Fig. 7. Intracellular sodium polyglutamate. Diffusion (see text).

Diffusion. The rate of diffusion, at comparable concentrations, was appreciably higher than that of the extracellular material (see Fig. 5, upper line). A similar C_i' against $Z_i^{\frac{1}{3}}$ behaviour was observed (Fig. 7). The extrapolated value at infinite dilution of $D_{20,w}$, again assuming a linear variation with j_m', was $1·91 \times 10^{-7}$ or $1·89 \times 10^{-7}$ uncorrected for the viscosity of the M-KCl–phosphate buffer.

Molecular weight. With the same value, 0·518, for the partial specific volume of the potassium salt, the molecular weight of the potassium salt is 96 000, equivalent to a value of 75 000 for the free acid. The frictional ratio, f/f_0 of the potassium salt is 4·1.

DISCUSSION

As already stated, the molecular weights calculated for the two specimens are valid provided only that any solvation, if present, is not selective. As Schachman & Lauffer (1950) and others have pointed out, sedimentation errors resulting from selective solvation can be greatly reduced by

carrying out measurements in dilute buffers, e.g. 0·1 M. In the present work, sedimentation measurements have been made down to such low concentrations that, even in 0·1 M salt, the error due to the primary charge effect would probably have been small. Diffusion measurements could not, however, be made at such low concentrations, and the linear extrapolation would be in some doubt (see, for example, Mandelkern & Flory, 1951).

The pronounced artificial-boundary sharpening observed in sedimentation and the marked dependence of S upon c are consistent with a particle having a large effective volume, and this is reflected in the high values for the frictional ratio. It appears likely from viscosity studies (to be reported elsewhere) that these high values of the frictional ratio are not associated with any marked asymmetry of shape.

The boundary-sharpening effect precludes any assessment of the degree of polydispersity of the specimens from the sedimentation and diffusion data.

SUMMARY

1. Electrophoresis, sedimentation and diffusion studies have been made on two samples of polyglutamic acid prepared from *Bacillus anthracis* grown *in vivo* and isolated from (a) the extracellular material in the blood of the animal and (b) from the intracellular material of the organisms.

2. Both specimens are shown to give only single boundaries in electrophoresis and sedimentation and have molecular weights of (a) 216 000 and (b) 75 000, expressed in terms of the free acid.

3. The specimens are typical polyelectrolytes with particles of large effective volume exhibiting large frictional ratios.

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The Polysaccharide from *Bacillus anthracis* Grown *in vivo*

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Bacillus anthracis contains polysaccharide material some of which appears to be part of the cell wall (Tomcsik, 1951; Chu, 1953). Ivanovics (1940a) first isolated a polysaccharide from *B. anthracis* which contained galactose 34%, glucosamine 34%, N 4·2% and α-carboxyl amino N 0·8%. Cave-Browne-Cave, Fry, El Khadem & Rydon (1954) obtained a polysaccharide preparation from *B. anthracis* which contained galactose 62%, glucosamine 32% and N 3·3%; they suggested that glucosamine residues may have been lost during their repeated treatment of this material with baryta. These authors also reported the isolation of a phosphorylated mannan from culture filtrates and from unwashed *B. anthracis*; this mannan could not be isolated from washed organisms. During the purification of a protective antigen from filtrates of cultures of *B. anthracis*, Strange & Belton (1954) obtained an inactive polysaccharide fraction which contained galactose 39–43%, glucosamine 34%, N 3·9% and α-carboxyl amino N 1·26%.

The differences between the total nitrogen and glucosamine nitrogen values, together with the figures for α-carboxyl amino nitrogen in these glucosamine-containing polysaccharides, indicated the presence of 5–10% of amino acid residues. Since the various preparations were not subjected to rigorous investigations of their purity, it was still undecided whether the amino acid residues were attached to the polysaccharide or were an impurity.