Molecular Structure of the Teichuronic Acid of *Bacillus megaterium*†

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ABSTRACT: The major component of the cell wall of *Bacillus megaterium* M46 was shown to be a complex polysaccharide over 20 times the size of previously reported teichuronic acids. The teichuronic acid was released from the sacculus by methods designed to preserve its structure intact and was purified to homogeneity by a combination of ion-exchange chromatography and gel filtration. The native material, in contrast to "typical" complex polysaccharides, was found to have a narrow size distribution and a compressed secondary structure. The molecular size and shape of this unusual teichuronic acid were elucidated by a combination of hydrodynamic, chemical, and electron microscopic studies. The polysaccharide elutes at the exclusion limit of Sepharose 2B and this exclusion is unaffected by the presence of a variety of common denaturants. The sedimentation and diffusion coefficients are strongly dependent upon concentration (k_{sed} = 240 cm^3/g; k_{dif} = 745 cm^3/g). Combination of the sedimentation coefficient at infinite dilution, $s_{20,w}°$ = 3.40 S, and the diffusion coefficient at infinite dilution, $d_{20,w}°$ = 0.47 × 10^{-7} cm^2/s, gives a ratio molecular weight of 490 000 ± 50 000. This value is supported by the number-average molecular weights obtained from the chemical determinations of the reducing groups (1 per 550 000 daltons ± 50 000) and the peptidoglycan attachment sites (1 per 500 000 daltons ± 50 000). The narrow size distribution was evident in the distribution of contour lengths from electron micrographs (85% of the molecules having a length within 15% of the mean value) and in the $G(s)$ distribution function (80% of the material having an $s_{20,w}°$ within 10% of the mean value). The high degree of asymmetry suggested by the concentration dependence of the sedimentation and diffusion coefficients was confirmed by viscometric studies. The limiting viscosity number of this polysaccharide is 450. The frictional ratio, 9.5, the viscosity increment, 360, and the value for the Scheraga and Mandelkern β parameter, 3.1, are all consistent with an equivalent hydrodynamic ellipsoid having an axial ratio of 70:1. The length of the molecule, 1250 Å, predicted from its hydrodynamic behavior was confirmed by electron microscopy. The teichuronic acid of *Bacillus megaterium* has a pitch per repeating tetrasaccharide of 1.6 Å, making it the most tightly packed polysaccharide so far reported.

The cell walls of gram-positive bacteria are composed of a peptidoglycan sheath with its associated proteins and teichoic and/or teichuronic acids. The precise function of these acidic macromolecules is at present not clear and, although they are generally regarded as essential components, they are often referred to as ancillary polymers. A knowledge of the structure of these ancillary polymers and of their structural relationships with the peptidoglycan should provide an understanding of the function of these components and a basis for studying the synthesis and assembly of the bacterial cell wall.

A continuously covalent cell envelope, sacculus, can be prepared from *Bacillus megaterium* M46 by detergent extraction combined with nucleolytic and proteinolytic digestion. These sacculi, which retain the shape of the bacillus, contain only two wall components: peptidoglycan and a polysaccharide composed of rhamnose, glucose, and glucuronic acid (White & Gilvarg, 1977). This uronic acid containing polymer is covalently linked to the peptidoglycan (Ivatt & Gilvarg, 1977), and thus fulfills both the compositional and structural criteria established for teichuronic acids. Uronic acid containing polymers have been isolated after acid extraction of the cell walls of several other species of gram-positive bacteria (*Bacillus licheniformis:* Janczera et al., 1961; *Micrococcus lysodeikticus:* Perkins, 1963; *Bacillus subtilus:* Ellwood & Tempest, 1972; *Corynebacterium:* Diaz-Maurino & Perkins, 1974) and are of low molecular weight. Other uronic acid containing polymers are secreted as capsular polysaccharides by gram-negative bacteria (Luderitz et al., 1968), and, although much more attention has been paid to their chemical properties rather than their physical properties, the general impression is that these latter polysaccharides are very large.

Since acid extraction of the cell wall is known to lead to a breakdown of the material under study (Hughes, 1970), the teichuronic acid of *Bacillus megaterium* M46 was isolated using procedures aimed at preserving the structure intact. The present study has provided the first correlation of hydrodynamic, chemical, and electron microscopic data for a bacterial polysaccharide and has revealed the intriguing finding that this teichuronic acid has the large size previously thought to be exclusively associated with secreted capsular polysaccharide.

Materials and Methods

Bacterial Strain and Growth Conditions. All experiments were carried out with *B. megaterium* M46. Conditions for growth and properties of the mutant have been described previously (Fukuda & Gilvarg, 1968; Pitel & Gilvarg, 1970).

Isolation of the Teichuronic Acid. Sacculi were prepared as described by White & Gilvarg (1977). The teichuronic acid was prepared by ion-exchange chromatography and gel filtration of the lysozyme digestion products of the sacculi as previously described (White & Gilvarg, 1977; Ivatt & Gilvarg, 1977). The polysaccharide was composed of the sugars rhamnose, glucose, and glucuronic acid in the proportions 2:1:1 and was substantially free of peptidoglycan, containing only a covalently linked tetrasaccharide dipeptide fragment which accounted for 0.4% of the total weight of the teichuronic acid.

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Ultracentrifugation. Sedimentation and diffusion studies with the purified teichuronic acid were performed at pH 6.8, in 0.1 M ammonium acetate in a Beckman Model E analytical ultracentrifuge. The temperature was maintained with an RTIC unit. Sedimentation velocity experiments were performed at 59 780 rpm at a temperature of 10 °C. Data for the determination of the sedimentation coefficient were obtained from schlieren photographs taken at 16-min intervals after reaching a speed of 59 780 rpm. Sedimentation coefficients were obtained from the limiting slope of the log x vs. time plots and corrected to a medium of water at 20 °C. The value of 0.64 mL/g for the partial specific volume was determined from the composition. The data were then analyzed in order to calculate $g(s)$ distribution functions of the sedimentation coefficients (Williams & Saunders, 1954), defined as follows:

$$g(s) = (1/C_0)(dc/ds)$$

where C_0 is the loading concentration, c is the concentration at a given radial distance and time, and s is the sedimentation coefficient associated with the radial distance and time. In terms of the parameters actually measured, the function has the following form:

$$g(s) = \omega^2 t(r^2/r_m^2)r(y_r)(1/y_0)$$

where ω is the angular velocity of the rotor in radians per second, t is the time in seconds (corrected for acceleration), r is the radial distance in centimeters, r_m is the radial position of the air-solution meniscus, the ratio (r^2/r_m^2) is a correction for radial dilution, y_r is the concentration gradient at radial position r, and y_0 is the total concentration of solute obtained from the area under the schlieren peak. The integral form of the distribution function, $G(s)$ is defined as

$$G(s_i) = (1/c_0)\int_0^{s_i} g(s)ds$$

and in terms of the parameters actually measured, has the following form:

$$G(s_i) = (1/y_0)\int_{r_m}^{r_i} (r^2/r_m^2)(y)dr$$

Data for the determination of diffusion coefficients were obtained from artificial boundaries formed by overlayering solutions of a slightly different concentration of teichuronic acid. Essentially symmetrical artificial boundaries were formed at a difference in teichuronic acid concentration of 0.2%. Schlieren photographs were taken at 8-min intervals after formation of the artificial boundary. The temperature was maintained at 20 °C.

Viscometric Studies. Viscosities were determined with a Zimm-Crothers viscometer (Zimm & Crothers, 1962) using a rotating Cartesian diver as suggested by Gill & Thompson (1967). The construction and use of this instrument have been described by Drlica & Worcel (1975). Viscosities of the teichuronic acid solutions were determined in 0.1 M ammonium acetate, pH 6.8, at 4 °C, by measuring the angular velocity of the rotor. The specific viscosity, η_{sp}, was obtained from the angular velocity of the rotor from

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} = \frac{t - t_0}{t_0}$$

where η is the viscosity of the sample, η_0 is the viscosity of the buffer, and t and t_0 are the revolution times of the rotor in sample and buffer, respectively. The limiting viscosity number was obtained from plots of ln η_{rel} vs. c, or η_{sp}/c vs. c.

Reducing Group Determination. The peptidoglycan fragment associated with the teichuronic acid was reduced with sodium [^{3}H]borohydride. Samples of the polysaccharide were treated overnight at 15 °C with 1% (w/v) sodium [^{3}H]borohydride (0.2 mL, 2 Ci/mol) in 0.1 M Tris-HCl[1] buffer, pH 8.4. The teichuronic acid was then extensively dialyzed against distilled water. A sample of muramic acid was reduced under identical conditions, acidified, and dried repeatedly from 0.1 N HCl. The teichuronic acid sample and the muramic acid standard were each dissolved in 1 mL of water and suspended in 10 mL of Bray's scintillant (Bray, 1960). Radioactivity measurements were made with a Packard Tri-Carb liquid scintillation spectrometer.

Electron Microscopy. Samples of the polysaccharide for the electron microscope were prepared in 0.1 M ammonium acetate, pH 6.8. Samples of 1 μL were air-dried onto carbon grids and stained with uranyl acetate [0.1% (w/v) in ethanol] for 20 s and then shadowed with platinum.

Results

Preparation of Teichuronic Acid. The teichuronic acid of *Bacillus megaterium* M46 was released from the cell wall by lysozyme digestion and purified by ion-exchange chromatography and gel filtration. The material is composed of rhamnose, glucose, and glucuronic acid in the molar ratio of 2:1:1, as previously described by White & Gilvarg (1977). This ratio is maintained across the elution profiles on both ion-exchange chromatography and gel filtration. The material was eluted at the exclusion limit of Sepharose 2B in 8 M urea, 0.2% Triton X-100, 1% $NaDodSO_4$, and 1 M hydroxylamine. This chromatography matrix has one of the largest exclusion limits of the gels commercially available. In order to obtain more than this minimum estimate of its size, sedimentation and diffusion studies were carried out.

Sedimentation Studies. Sedimentation velocity experiments were performed in 0.1 M ammonium acetate, pH 6.8. A single, extremely sharp sedimentation boundary was obtained at a concentration of 1.0%. During the run, there was very little broadening of the boundary—it appeared as sharp after 3 h of sedimentation as at the beginning. This would appear to be the behavior of material possessing a high degree of molecular homogeneity and a low diffusion coefficient. However, on repeating the experiment with a 0.1% solution, the boundary showed broadening with time, and the sedimentation took place nearly three times as rapidly. The sedimentation profiles of the teichuronate at 0.6% and 0.3% are shown in Figure 1A. They illustrate self-sharpening and broadening boundaries, respectively. It is apparent that there is a considerable departure from ideal solution laws. In order to obtain a reliable sedimentation coefficient for the calculation of the molecular weight, the relationship between sedimentation coefficient and concentration was studied. The nonideality was corrected for by extrapolating to infinite dilution.

There was a tendency for the sedimentation coefficient (determined at intervals during a run) to increase as the run progressed. This was due in part to a progressive dilution of the polysaccharide solution, resulting from the sector shape of the cell. However, it was not possible to obtain a well-defined drift in the calculated sedimentation coefficient during a run, and so a mean value has been determined over the duration of each run. This value refers strictly to a solution of slightly lower concentration than that placed in the cell at the beginning of an experiment. The effect of this error on the extrapolated value at infinite dilution is negligible and has been ignored. The

[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

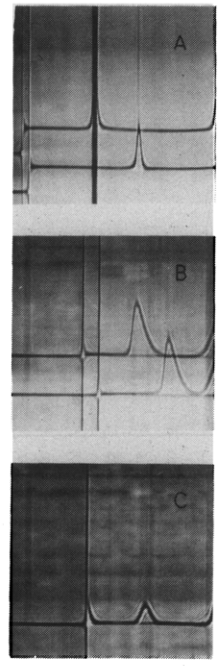

FIGURE 1: Sedimentation and diffusion boundaries of the teichuronic acid. Photograph A shows the sedimentation boundaries of teichuronic acid samples centrifuged in 0.1 M ammonium acetate, pH 6.8. The polysaccharide concentrations of the samples are 0.6% (w/v) (upper boundary) and 0.3% (w/v) (lower boundary). Migration of the boundaries is from left to right. The photograph was taken 90 min after reaching a speed of 59 780 rpm. The temperature of the experiment was 5 °C, and the Schlieren plate angle was 70°. Photographs B and C show diffusion boundaries of teichuronic acid samples obtained in 0.1 M ammonium acetate, pH 6.8. The boundaries shown in photograph B were formed by layering solvent onto solutions of the polysacharide. The concentrations of the polysaccharide solutions were 1.0% (w/v) (upper boundary) and 0.8% (w/v) (lower boundary). The boundary formed shown in photograph C was formed by layering the 0.8% (w/v) polysaccharide solution over the 1.0% (w/v) polysaccharide solution. Photographs were taken 60 min after boundary formation with a Schlieren plate angle of 70°C.

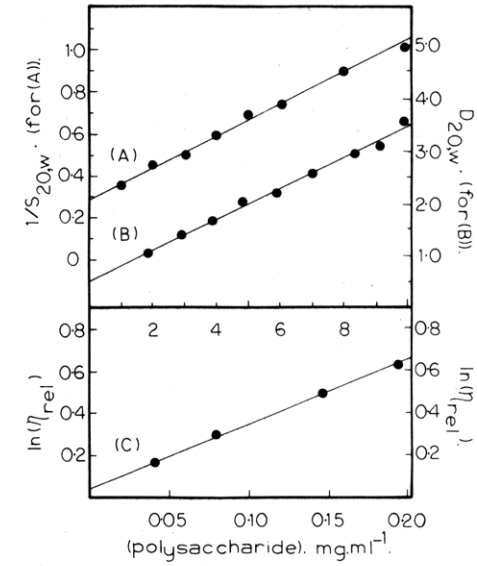

FIGURE 2: Concentration dependence of the sedimentation coefficient, diffusion coefficient, and relative viscosity of the teichuronic acid. (A) Sedimentation velocity experiments were performed at different teichuronic acid concentrations under the conditions described in the legend to Figure 1A. The reciprocals of the sedimentation coefficients, corrected to water at 20 °C, are plotted against the polysaccharide concentration at which they were obtained. (B) Diffusion coefficients were obtained at different teichuronic acid concentrations, from artificial boundaries formed at a concentration difference of 0.2% (w/v), under the conditions described in the legend to Figure 1C. The diffusion coefficients, corrected to water at 20 °C, are plotted against the polysaccharide concentration at which they were obtained. (C) The relative viscosity of the teichuronic acid was determined at different polysaccharide concentrations, from the rotation times observed with a Zimm–Crothers viscometer. Viscosities were determined in 0.1 M ammonium acetate, pH 6.8, at 4 °C. The logarithm of the relative viscosity is plotted against the polysaccharide concentration at which it was obtained.

sedimentation coefficients, obtained as above, have been corrected to a medium of water at 20 °C. There was a linear relationship between the reciprocal of the sedimentation coefficient and the concentration of teichuronate.

The limiting value of the sedimentation coefficient, $s_{20,w}°$, was 3.40 S. This value was obtained from the reciprocal of the intercept at zero teichuronate concentration (Figure 2A). The second consequence of this large variation in sedimentation coefficient with concentration is seen in the exceptionally sharp sedimentation boundaries, except with the most dilute solutions of the teichuronate. This sharpening is caused by the smaller molecules, which would naturally lag behind the main boundary, having an increased sedimentation velocity due to the low concentration in that region and the larger particles, migrating in a region of high concentration, having a relatively small sedimentation velocity. This effect calls for caution in deducing the degree of polydispersity from the rate of boundary spreading.

Diffusion Studies. The large increase in the sedimentation coefficients with diminishing concentration indicated an apparent increase in the "effective" molecular weight of the teichuronate on dilution. A similar effect, in the same direction, was found with the diffusion coefficient. This is illustrated by the asymmetric artificial boundaries shown in Figure 1B. The material is duffusing faster in the solution than in the solvent, resulting in a concentration gradient that is steeper on the solvent side than on the solution side of the boundary. In order to follow the variation in the diffusion coefficient with concentration and at the same time to avoid the difficulty of dealing with asymmetric concentration distributions, one solution of the teichuronate was layered over another teichuronate solution of slightly different concentration. The concentration difference was kept at 0.2%, and practically symmetrical boundaries were obtained (Figure 1C). The calculated diffusion coefficients were corrected to diffusion in pure water. The results are shown in Figure 2B. The diffusion coefficients show a considerable increase with increasing concentration. There is a linear relationship between the diffusion coefficient and the teichuronic acid concentration. The limiting value of the diffusion coefficient, $D_{20,w}$, was 0.47×10^{-7} cm^2 s^{-1}. This value was obtained from the intercept at zero teichuronate concentration.

Chemical Studies. The size of the teichuronic acid was determined by measuring the average weight of the teichuronic acid per reducing terminal. The number of reducing terminals was determined by following the incorporation of radioactivity into the teichuronic acid and into a muramic acid standard during reduction with sodium [^{3}H]borohydride. The results are presented in Table I. The specific activity of the [^{3}H]-teichuronic acid indicates that there is one reducing terminal for every 875 glucuronic acid residues, giving an average molecular weight of 550 000. It should be noted that previous studies had established that better than 90% of the teichuronate chains terminated in a tetrasaccharide peptidoglycan fragment (Ivatt & Gilvarg, 1977).

Viscometric Studies. One of the most informative hydrodynamic properties regarding the shape of a molecule is the intrinsic viscosity. The viscosities of teichuronic acid solutions

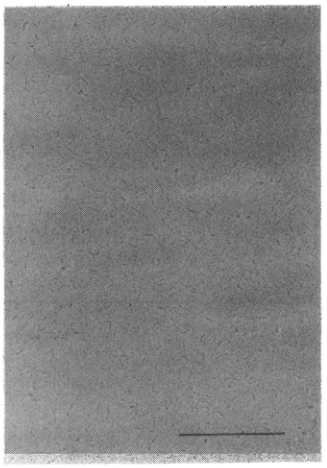

FIGURE 3: Electron microscopic appearance of the teichuronic acid. A teichuronic acid sample (1 μL) equilibrated against 0.1 M ammonium acetate, pH 6.7, was air-dried onto a carbon grid and stained with uranyl acetate (0.1%, w/v, in ethanol) and shadowed with platinum. The bar is 1.0 μm in length.

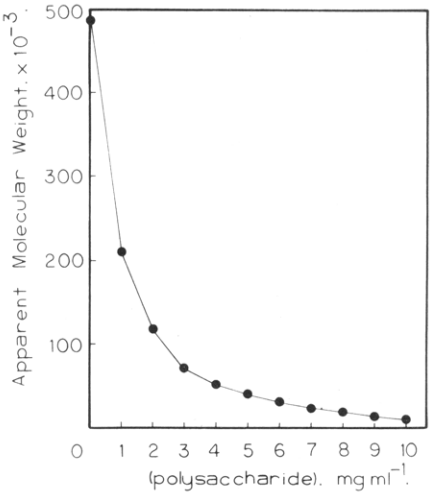

FIGURE 4: Concentration dependence of the ratio molecular weight of the teichuronic acid. Sedimentation and diffusion coefficients determined at different polysaccharide concentrations were combined in the Svedberg equation to provide ratio molecular weights. These ratio molecular weights are plotted against the polysaccharide concentrations at which the sedimentation and diffusion coefficients were determined.

TABLE I: Average Weight of the Teichuronic Acid per Reducing Terminal.[a]

	teichuronate	muramate
^{3}H incorp (cpm)	2.17×10^4	2.29×10^6
sp act. (cpm/μmol)	8.16×10^{2}[b]	7.17×10^{5}[c]
μmol/μmol of reduc group	879[b]	1[c]
wt/mol of reduc group	554 000[d]	293

[a] Samples of the teichuronic acid (containing 26.6 μmol of glucuronic acid) and the muramic acid standard (3.2 μmol) were reduced with sodium [^{3}H]borohydride as described under Materials and Methods. [b] Micromole(s) of glucuronate. [c] Micromole(s) of muramate. [d] Based on a molecular weight of 630 for repeating unit.

were determined in 0.1 M ammonium acetate, pH 6.8, at 4 °C. The angular velocity of the rotor was independent of the number of rotations, demonstrating the independence of the viscosity to shear stress. The average shear stress in the standard assay was 8×10^{-3} dyn cm^{-2} (calculated by the method of Zimm & Crothers, 1962). The viscosity determined at different polysaccharide concentrations was plotted, either as ln η_{rel} vs. concentration (Figure 2C) or as η_{sp}/c vs. concentration. The former plot was smoother, and the value extrapolated to zero teichuronate concentration gave a limiting viscosity number of 450.

Electron Microscopic Appearance of the Teichuronic Acid. The teichuronic acid has a rigid filamentous appearance when spread from 0.10 M ammonium acetate (Figure 3). The ability of the molecules to pick up uranyl atoms was very variable. Occasionally, long extended tangles of the polysaccharide were observed, similar to the aggregates described by Hanke & Northcote (1975), with polygalacturonic acid. However, these aggregates occur during the preparation of the electron-microscope grids and are not related to the usual solution conformation. They usually include all the visible polysaccharide molecules and measure more than 10 μm. Since the samples were filtered on a 0.1-μm Millipore filter prior to spreading, these large aggregates are presumed to form in the hypophase.

Discussion

The acidic polysaccharide of *Bacillus megaterium* M46 has been shown to be covalently associated with the cell-wall peptidoglycan and is therefore a teichuronic acid (Ivatt & Gilvarg, 1977). The present study has revealed several unusual aspects of this teichuronic acid. Firstly, it has an unusually high molecular weight. Secondly, the narrow size distribution suggests an unusually precise coordination of chain initiation and termination. Finally, the large difference between the extended chain length for this polysaccharide and its observed molecular shape suggests a well-ordered structure.

Molecular Size of the Teichuronic Acid. The high molecular weight of the teichuronic acid, suggested by its exclusion from Sepharose 2B, was confirmed by sedimentation and diffusion studies. These hydrodynamic studies were complicated by the high degree of nonideality usually experienced with large asymmetric macromolecules. Therefore, the values of the sedimenation and diffusion coefficients used in the calculation of the ratio molecular weight were obtained by extrapolation to zero concentration. There was a linearly inverse relationship between the sedimentation coefficient and concentration; the concentration dependence of the sedimentation coefficients K_{sed} was 240 cm^3 g^{-1}. For the diffusion coefficient, there was a linear relationship between it and the concentration. The concentration dependence of the diffusion coefficient k_{dif} was 745 cm^3 g^{-1}. The values for the sedimentation and diffusion coefficients, extrapolated to zero concentration, were 3.40 S and 0.47×10^{-7} cm^2 s^{-1}. A ratio molecular weight of 490 000 was obtained for the teichuronic acid from the Svedberg equation, using these values for the sedimentation and diffusion coefficients and a value of 0.64 cm^3 g^{-1} for the partial specific volume.

Since this value for the molecular weight is large, relative to previously studied ancillary polymers, it was important to establish that this size was not the result of self-aggregation phenomena. The concentration dependencies of the sedimentation coefficient and diffusion coefficient were both in the contrary direction expected for a self-aggregating system; viz., the sedimentation coefficient was found to increase with decreasing concentration, while the diffusion coefficient was found to decrease with decreasing concentration, both k_{sed} and k_{dif} had large positive values. The apparent molecular weight, determined from the $s_{20,w}$ and $D_{20,w}$ at a particular concentration, rapidly decreases with increasing concentration (Figure 4), making it unlikely that the large molecular size is

TABLE II: A Summary of the Data Related to the Size and Shape of the Teichuronic Acid.

sedimentation coeff[a]	$s_{20,w}°$	3.40 S
diffus coeff	$D_{20,w}°$	0.47×10^{-7} cm^2 s^{-1}
mol wt		
from Svedberg eq	M_r	488 000
per peptidoglycan attach. site[b]	M_n	500 000
per reducing terminal	M_n	550 000
limiting viscosity no.	η	450 cm^3 g^{-1}
concn dependence of $s_{20,w}°$	k_s	240 cm^3 g^{-1}
concn dependence of $D_{20,w}°$	k_d	745 cm^3 g^{-1}
frictional ratio	f/f_0	9.5
viscosity increment[c]	ν	360
parameter[d]	β	3.1
axial ratio	R	70:1
length from electron micrograph	L	1240 Å
length from viscosity increment[e]	L	1000 Å

[a] Extrapolated to infinite dilution. [b] From Ivatt and Gilvarg (1977). [c] Einstein-Simha (1940). [d] Scheraga and Mandelkern (1953). [e] Flory (1953).

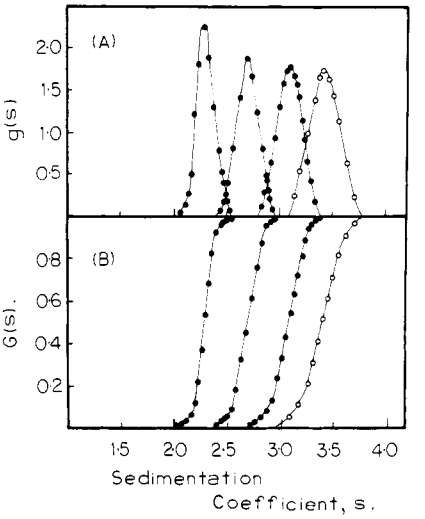

FIGURE 5: Sedimentation coefficient distribution functions of the teichuronic acid. The graph shows the $g(s)$ curves (upper profiles) and $G(s)$ curves (lower profiles) of teichuronic acid samples. The curves were calculated from sedimentation boundaries of the polysaccharide obtained from experiments performed at polysaccharide concentrations (solid symbols; from left to right) of 0.2%, 0.1%, and 0.05% (w/v). The $g(s°)$ and $G(s°)$ curves obtained by extrapolation to account for nonideality are also shown (open symbols).

the result of a reversible association (dissociation with increasing concentration!) and emphasizing the need to obtain data over as large a concentration range as possible. The possibility that the molecular entity with molecular weight 490 000 is the result of a noncovalent aggregation of lower molecular weight subunits is also unlikely for the following reasons. Firstly, if the molecular-weight form studied in this investigation is a stable component of an aggregating system, there should be an observable perturbation of the system by the addition of denaturants or by changes in the pH or ionic strength of the solution. This was not observed. The material was eluted at the exclusion limit of Sepharose 2B in the presence of a variety of commonly used denaturants, over a range of pH values (3.0–7.0) and over a range of ionic strengths. It thus maintained a hydrodynamic radius of 300 Å, or greater, under all these conditions. Secondly, the low reducing sugar content found with this teichuronic acid is consistent with there being one reducing group per 550 000 daltons of polysaccharide. This is consistent with the third line of evidence, namely, the proportion of peptidoglycan associated with the teichuronic acid after extensive lysozyme digestion and β elimination, which indicated the presence of one attachment site to the peptidoglycan per 500 000 daltons of polysaccharide (Ivatt & Gilvarg, 1977). The data regarding the molecular size of the teichuronic acid are summarized in Table II. It is therefore reasonable, on the basis of the aggreement in molecular-weight determination based on the hydrodynamic and chemical data, to regard the teichuronic acid as a discrete molecule with a molecular weight of 490 000 daltons. The molecular weight of 490 000 makes the teichuronic acid from *Bacillus megaterium* over 20 times the size of previously described teichuronic acids (*Bacillus licheniformis:* Hughes, 1970; *Micrococcus luteus:* Nasir-ud-Din and Jeanloz, 1976) and teichoic acids (Baddiley, 1972). Indeed, this large size is comparable to the reputed size of the capsular polysaccharides secreted by *Escherichia* (Bayer & Thurow, 1977), *Pneumococcus* (Larm & Lindberg, 1976), and *Klebsiella* (Luderitz et al., 1968), and it should be noted that, while these capsular polysaccharides are undoubtedly of high molecular weight, their physical properties have been neglected in favor of compositional studies, and this impression of their having a large size is largely based upon gel-permeation chromatography. Structural studies (Ivatt & Gilvarg, 1977) have clearly demonstrated that this polysaccharide is covalently linked to the peptidoglycan and is therefore an integral part of the cell wall and not a secreted capsule. The usual distinction between wall and capsule is not appropriate for *Bacillus megaterium* as its teichuronic acid contributes a polysaccharide layer around the organism that is morphologically identical to a capsule (Ellar et al., 1967). It is therefore an intriguing situation: A polysaccharide that functions as capsular material yet is synthesized and assembled into the cell wall as a conventional wall polymer. The other noteworthy aspect of the cell wall of this strain of *B. megaterium* is that it lacks the ordered protein layer observed by Holt & Leadbetter (1969) in a wide variety of other *Bacillus* species. A low level of associated protein (Hitchins & Gilvarg, unpublished observation) and the absence of other wall polymers (White & Gilvarg, 1977) make this strain ideal for studying the function and assembly of teichuronic acids.

Molecular-Weight Distribution of the Teichuronic Acid. There are several lines of evidence which suggest that the molecular-weight distribution of the teichuronic acid is fairly narrow: firstly, the very good correspondence between the number-average molecular weight determined chemically and the ratio molecular weight obtained from the hydrodynamic studies; secondly, there is a fairly narrow distribution of sedimentation coefficients, 80% of the material having a sedimentation coefficient within 10% of the mean value; thirdly, the narrow distribution of lengths measured on the electron micrographs, over 85% of the polysaccharide molecules have a length within 15% of the mean value.

The $g(s)$ method used in the present work was first used with polysaccharides by Williams & Saunders (1954) and has proved to be a very powerful approach for the study of the protein–polysaccharide complex from nasal cartilage in the hands of Sajdera & Hascall (1969, 1970). Plots of the $g(s)$ distribution function vs. s are shown in Figure 5A, at 0.2%, 0.1%, and 0.05% teichuronic acid. The $g(s°)$ vs. s curve corresponding to infinite dilution is also shown. The corresponding $G(s)$ vs. s curves are shown in Figure 5B and reveal that 80% [$0.1 < G(s°) < 0.9$] of the material has a sedimentation

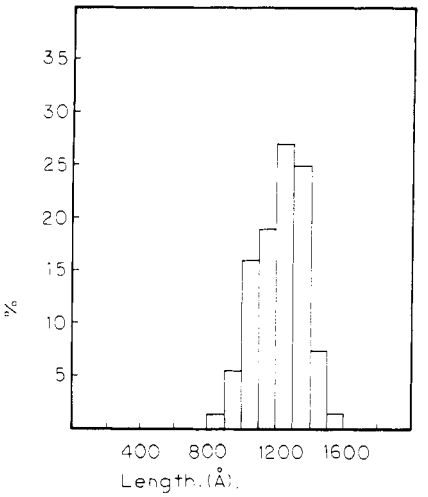

FIGURE 6: Distribution of contour lengths from electron microscopy. The lengths of the teichuronic acid molecules were measured on the original negative of the field shown in Figure 3, with a Nikkon microcomparator. A total of 150 molecules were measured.

coefficient within 10% of the mean ($3.1_6 < s° < 3.6_2$). This distribution is consistent with the distribution of contour lengths measured from the electron micrographs (Figure 6). The number, weight, and z-average lengths are 1221, 1240, and 1260 Å, respectively. The ratio of 1.02 for adjacent averages is clearly indicative of a tight distribution. Polysaccharides are synthesized by a template-independent polymerization, so that the length of the polymer is determined by the interval between initiation and termination. For the present teichuronic acid to have a relatively tight distribution of lengths, the mechanisms that regulate initiation and termination must be very closely coordinated.

Molecular Shape of the Teichuronic Acid. The hydrodynamic behavior of a molecule is a complex function of its size, shape, solvation, flexibility, and other properties. The problem of giving solvation and other ill-defined factors explicit consideration was avoided by Scheraga & Mandelkern (1953), who defined an equivalent hydrodynamic ellipsoid of revolution. The requirement for the determination of the axial ratio of this ellipsoid was a knowledge of the molecular weight and of two hydrodynamic parameters which are sensitive to different powers of the molecular size, usually the sedimentation coefficient ($s°$) and the intrinsic viscosity (η). The value of β, the Scheraga & Mandelkern parameter, obtained for the teichuronic acid in the present investigation (using values of 490 000 for the molecular weight, 3.40 S for the sedimentation coefficient, and 450 $cm^3\ g^{-1}$ for the intrinsic viscosity) was 3.1. According to the data tabulated by Scheraga and Mandelkern (1953), this value of β corresponds to a prolate ellipsoid with an axial ratio of 70:1. This high degree of asymmetry is supported by the high value of the viscosity increment. Einstein has shown that for solid impenetrable spheres, the viscosity increment is 2.5. The much larger value obtained in the present work (360) is indicative of a high degree of molecular asymmetry and yields a contour length of 1000 Å for the polysaccharide (Flory, 1953). The value of 0.53 for the ratio of the concentration dependence of the sedimentation coefficient to the limiting viscosity number, k_{sed}/η, is much less than the theoretical value of spherical molecules (Wales & Van Holde, 1954; Yamakawa, 1962) of 1.6 and is near the values of known elongated molecules, 0.23 for calf-skin collagen (Nishihara & Doty, 1958) and 0.34 for rabbit-muscle myosin (Holtzer & Lowey, 1959). A third aspect of the hydrodynamic behavior that is consistent with the teichuronic acid being a highly asymmetric molecule is the frictional ratio. This ratio, of the frictional coefficient obtained from the diffusion data to the frictional coefficient calculated for a sphere with equivalent molecular weight, is indicative of the shape of the molecule. However, there are uncertainties with this ratio in that there is a need to consider solvation and other solution effects. In the present investigation, the frictional coefficient for a sphere of equivalent weight was obtained from the data available for dextran particles (Granath, 1958; Laurent & Granath, 1967). These particles have the hydrodynamic properties and the electron microscopic appearance of spherical molecules, and, being polysaccharides, they are good analogues for solvation and other solution effects. The frictional ratio obtained on this basis was 3.5. By comparison in the absence of these considerations, the frictional ratio can be calculated to be 9.5, using a partial specific volume of 0.64 $cm^3\ g^{-1}$, and a molecular weight of 490 000. This ratio is a comparison of the empirically determined properties with those of a rigid, impenetrable, anhydrous sphere of identical molecular weight. The hydrodynamic studies provide compelling evidence that the teichuronic acid is a large, highly asymmetric molecule, whose behavior is described by a prolate ellipsoid of molecular weight 490 000 and with an axial ratio of 70:1. The data regarding the molecular shape of the teichuronic acid are summarized in Table II. This model derived from the hydrodynamic data is largely confirmed by the appearance of the polysaccharide in electron micrographs; the asymmetric rods have contour lengths in very good agreement with the length calculated from the viscosity increment.

The molecular structure of the teichuronic acid suggests a much more compressed secondary structure than has usually been presented for complex polysaccharides. The capsular polysaccharide of *E. coli* serotype K29 strain, with which the present teichuronate might be expected to be analogous, has a pitch per residue of 3.8 Å (Moorehouse et al., 1977), resulting in a relatively extended structure. This value for the pitch per sugar residue is close to the 4.3 Å obtained for ι-carrageenan (Arnott et al., 1974a), the 4.2 Å obtained for hyaluronic acid (Guss et al., 1975), and the 3.2 Å obtained for agarose (Arnott et al., 1964b). In contrast, the present teichuronic acid has a pitch per residue of 0.5 Å (as only three sugars per repeating unit are in the main backbone, Ivatt and Gilvarg, unpublished observation) and therefore more closely resembles amylose, 1.3 Å pitch per residue (French, 1973; Hybl et al., 1965), than the more extended capsular polysaccharide and predicts a much wider, more slowly rising helix for the teichuronic acid. Another aspect revealed by these earlier studies is the occurrence of multiple-strand helices for several of these polysaccharides (ι-carrageenan, agarose, and amylose) and also for the xylans from algal cell walls (Preston, 1968) and the xanthan from *Xanthmonas campestris* (Holzwarth and Prestridge, 1977). These structures have been eliminated for the teichuronic acid due to the good agreement between the molecular weights determined chemically and hydrodynamically; i.e., each large rod 1200-Å long and weighing 490 000 daltons is composed of a single polysaccharide chain. The present studies have revealed the major component of the cell wall of *Bacillus megaterium* M46 to be an unusual molecule, the information concerning its size and shape forms a solid base for exploring the organization of the cell wall and the mechanism of its assembly.

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