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TECHNICAL MANUSCRIPT 487

FURTHER INVESTIGATIONS ON THE MOLECULAR WEIGHT OF STAPHYLOCOCCAL ENTEROTOXIN B

David Trkula
Robert L. Sine Jr.
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SEPTEMBER 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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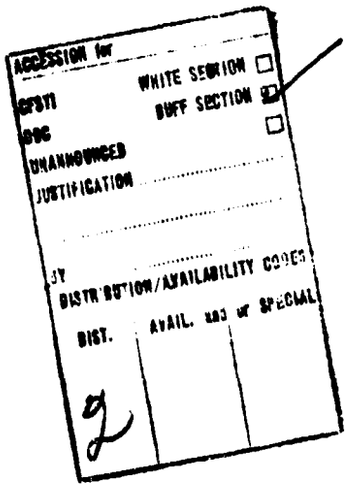

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Project 1B56303D164

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ABSTRACT

The molecular weight of staphylococcal enterotoxin B was determined independently and by methods not previously applied to the protein; the results agree well with those obtained in two earlier studies. In order to check the previous determinations, analyses were performed in 0.05 M sodium phosphate buffer, pH 6.8, and in 0.1 ionic strength sodium Veronal buffer, pH 8.6, the isoelectric point of the protein. The sedimentation and diffusion method yielded 33,000±1,400 daltons in Veronal buffer and 31,800±1,000 daltons in phosphate buffer. The Yphantis meniscus-depletion sedimentation equilibrium method yielded average values of 31,000±1,300 daltons in Veronal and 30,400±2,000 daltons in phosphate. Osmometry yielded an average value of 26,800±300 daltons in Veronal buffer.

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I. INTRODUCTION

The molecular weight of purified staphylococcal enterotoxin B has been experimentally determined twice previously. The first determination was by Wagman, Edwards, and Schantz,[1] who reported a value of 35,300 by sedimentation and diffusion (free boundary diffusion in an analytical ultracentrifuge) and a value of 35,100 by the Archibald approach-to-equilibrium method; these analyses were done in 0.05 M potassium phosphate buffer at pH 6.8. The second determination was by Bergdoll et al.,[2] who performed their experiments in 0.1 M Veronal buffer at pH 8.6, the isoelectric point of the protein. They reported a value of 30,777 by sedimentation and diffusion; the diffusion analysis was carried out in agar by the method described by Schantz and Lauffer.[3]

The purpose of the present investigation was to attempt to resolve the discrepancy in the molecular weights reported in the two preceding investigations. To this end, the molecular weights were determined by sedimentation and diffusion, sedimentation equilibrium, and osmometric methods. Experiments were conducted in sodium phosphate buffer, pH 6.8, and in sodium Veronal buffer at the isoelectric point, pH 8.6. Thus, it was possible to check the previous determinations by repeating measurements in the two media and by making other, different measurements.

II. MATERIALS AND METHODS

A. THE PROTEIN

Solutions of staphylococcal enterotoxin B were prepared by dissolving 10- or 100-mg amounts of lyophilized protein contained in vials in buffer. The lyophilized protein had been purified by chromatography on CC-50 resin and CM-cellulose, as described by Schantz et al.[4] The protein solutions were, in most cases, filtered through Millipore filters (0.45-μ pores) prior to concentration determinations and experimental operations. The buffers and water used as solvents were routinely filtered through 0.22-μ or 0.45-μ Millipore filters prior to use. Lot C-40 protein was used only in a series of sedimentation experiments as a comparison with lot C-41, used in all the other experiments reported here. The protein solutions were routinely stored in the refrigerator at about 5 C.

B. CONCENTRATION

Concentration of protein was determined by UV absorption at 277 mμ and/or by Kjeldahl nitrogen analysis. The extinction coefficients used in the UV analyses were 14 in 0.05 M phosphate[4] and 14.5 in 0.1 ionic strength sodium Veronal, for a 1% protein solution and a 1-cm path length. The factor used for conversion of nitrogen to protein concentration was 6.21, based on a nitrogen content of 16.1%.[4]

C. ELECTROPHORESIS

Electrophoretic analyses were conducted in a Perkin-Elmer Model 238 Electrophoresis Apparatus using schlieren optics and standard 15-mm cells. The solutions were analyzed at a concentration of 5 mg/ml, following dialysis in three changes of the suspending buffers (0.1 ionic strength sodium Veronal and 0.1 ionic strength sodium borate, both at pH 8.6). The boundaries were formed against the final dialysate. The analyses were carried out at about 5 C. The current of 4 milliamps was maintained for as long as 8 hours. Photographs were taken on the Polaroid camera furnished with the instrument.

D. SEDIMENTATION

Velocity sedimentation runs were performed in a Spinco Model E analytical ultracentrifuge using the standard schlieren optical system equipped with a phase plate. The analyses were done in 0.05 M sodium phosphate and 0.1 ionic strength sodium Veronal buffers at pH 6.8 and pH 8.6, respectively, at protein concentrations between 1 and 10 mg/ml. All runs were conducted at 59,780 rpm using a rubber valve-type, aluminum synthetic boundary cell, with 4 degree sector and 12 mm thickness, to facilitate formation of the boundary.

Sedimentation coefficients were calculated from displacements of maximum ordinates of the gradient curves as functions of time, according to the method described by Schachman.[5] The reference distances were obtained by measurement of distances of the counterbalance reference edges from the rotor axis on a Gaertner Microcomparator. Reference distances were corrected for rotor stretch at 59,780 rpm according to the method described by Schachman.[5]

E. DIFFUSION

Diffusion analyses were done according to the method described by Schumaker and Schachman[6] and by Pedersen.[7] UV absorption optics were employed in the Spinco Model E analytical ultracentrifuge with recording

on Kodak Commercial film. One diffusion run was performed in 0.05 M sodium phosphate buffer, pH 6.8, at 4 C. The other diffusion experiment was carried out in 0.1 ionic strength sodium borate buffer, pH 8.6, at 20.0 C. This experiment would have been done in Veronal buffer, except that the buffer absorbs at 254 mμ, the principal wavelength of the UV lamp on the centrifuge. The concentration of enterotoxin was 1.2 mg/ml in both experiments; the protein had been dialyzed three times against the suspending buffer. The salt content of the protein solution was increased by 0.025 M with KCl to prevent boundary disturbance when the overlay buffer was deposited in the valve-type synthetic boundary cell. The overlay buffer was the third dialysate in both cases. Both experiments were done at 21,740 rpm. A double-beam recording microdensitometer* was used to convert the film images into traces of optical density as a function of position in the centrifuge cell.

Diffusion coefficients were calculated by dividing the slope of the plot of u_n^2 $(1 - \omega^2 St)$ versus t by 3.64. The radial distance between the 25% and 75% optical density points in the boundary region is u_n, t is time in seconds, and $(1 - \omega^2 St)$ is the correction for rotor speed, where ω is angular velocity of the rotor, and S is the sedimentation coefficient of the protein under the conditions of the diffusion experiment. The calculated diffusion coefficient at 4 C was corrected to 20 C by use of the relationship

$$D = D' \frac{T \eta_w'}{T' \eta_w}$$

where D' is the calculated diffusion coefficient at Kelvin temperature T' (277.16 K), D is the diffusion coefficient corrected to 20 C (T = 293.16 K), η_w' is the viscosity of water at 4 C, and η_w is the viscosity of water at 20 C.

F. BUOYANCY FACTOR

Buoyancy factors $(1 - \bar{V}\rho)$ were determined by pycnometry in 10- and 20-ml Lipkin pycnometers** at protein concentrations ranging between 2 and 18 mg/ml in water, sodium phosphate buffer (0.05 M, pH 6.8) and sodium Veronal buffer (0.1 ionic strength, pH 8.6) at 20.0±0.1 C. Protein concentrations were determined by Kjeldahl nitrogen analyses. The nitrogen values for protein in Veronal were corrected by subtracting the value for nitrogen determined on a Veronal blank.

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* MKIIIB, Joyce, Loebl and Co., Ltd.
** Ace Glass Co., Vineland, N.J.

The pycnometers were calibrated with water. Weighing was on an Ainsworth Type SCN balance or a Voland Universal Analytical Balance, Model 860D. Buoyancy corrections were applied to all weights. Weights of pycnometer contents were converted to a pycnometer capacity of 1 ml for the calculations.

In the determination of $(1 - \bar{V}\rho)$ in water, the protein was prepared by dialysis against distilled water, and the successive concentrations of protein were prepared by dilution of the dialyzed protein stock with filtered distilled water; filtered distilled water was used for the zero protein concentration point (Fig. 1). For the determination in Veronal buffer, the stock protein was prepared by dialysis against stock Veronal buffer, and the successive concentrations of protein were prepared by dilution of the dialyzed protein stock with stock Veronal buffer; the density of stock Veronal buffer was used as the zero protein concentration point (Fig. 2). For the $(1 - \bar{V}\rho)$ determination in phosphate buffer, the protein solutions were prepared in two ways. In one experiment, stock protein solution was prepared by dialysis against stock phosphate buffer, and successive dilutions were made in stock phosphate buffer; the density of stock phosphate buffer was used as the zero concentration point. In the other experiment, stock protein solution was prepared by dialysis against stock phosphate buffer. Successive concentrations of protein were, however, prepared by dilution of the dialyzed non-isoelectric protein stock solution with dialysate;[8] the density of the dialysate buffer solution was used as the zero concentration point in the graphical estimation of $(1 - \bar{V}\rho)$ (Fig. 3).

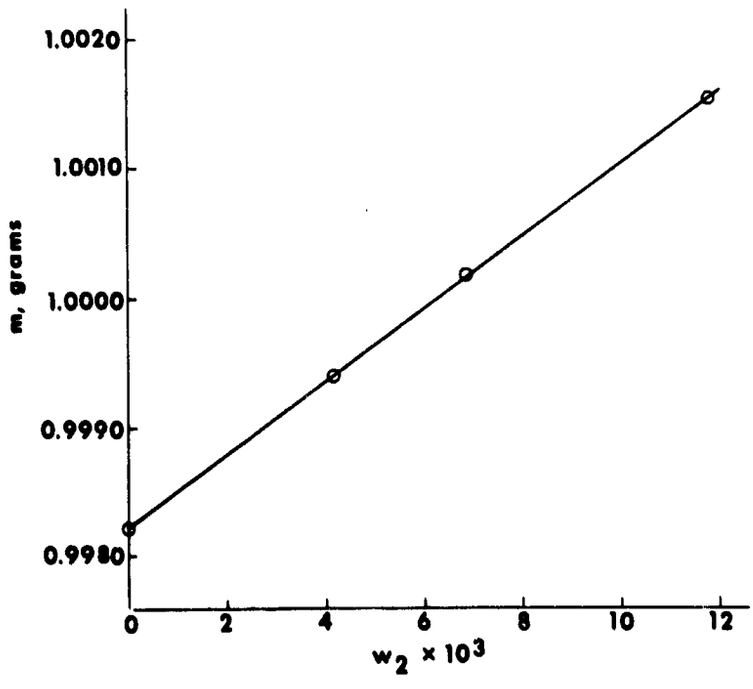

Figure 1. Plot of Mass of Pycnometer Contents (Normalized to 1 ml) Against Weight Fraction, w_2, of Staphylococcal Enterotoxin B in Distilled Water.

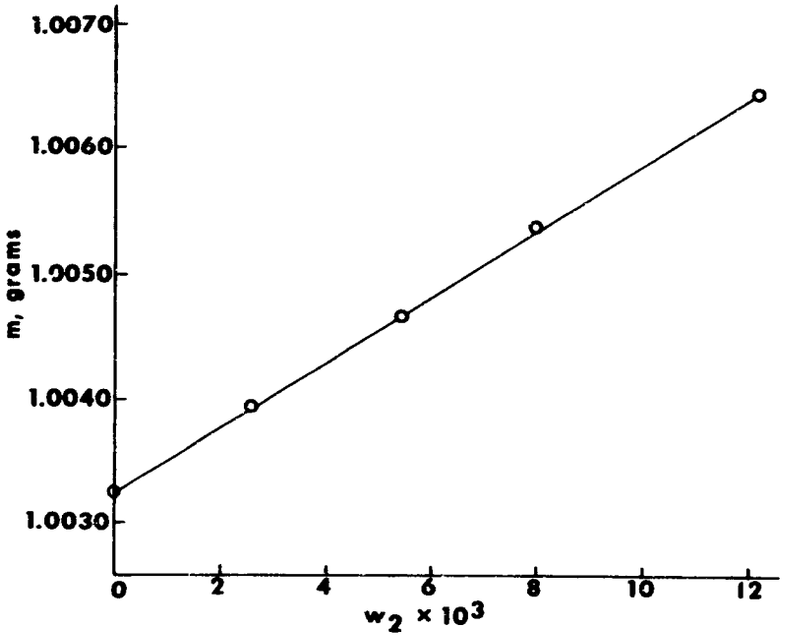

Figure 2. Plot of Mass of Pycnometer Contents (Normalized to 1 ml) Against Weight Fraction, w_2, of Staphylococcal Enterotoxin B in 0.1 Ionic Strength Veronal Buffer, pH 8.6.

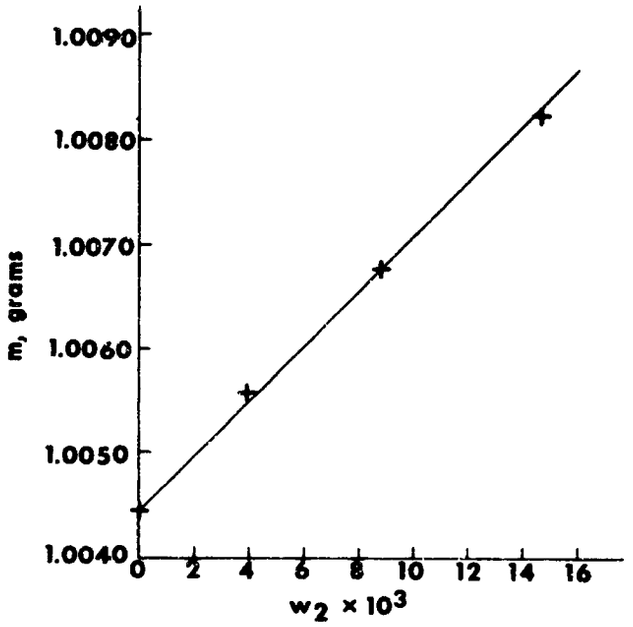

Figure 3. Densities of Staphylococcal Enterotoxin B Protein in Dialysate Phosphate Buffer at 20 C. The stock phosphate buffer was 0.05 M, pH 6.8. m is mass of pycnometer contents normalized to a 1-ml volume, w_2 is weight fraction of the toxin protein in dialysate buffer.

Buoyancy factors were calculated according to the procedure described by Kay[8] and by Kraemer.[10] Weight of pycnometer contents, m, is plotted versus weight fraction, w_2, of protein solution. If a straight line is obtained (as was the case in all our experiments, Figures 1 through 3), the buoyancy factor for sedimentation, $(1 - \bar{V}\rho)$, is evaluated by multiplying the slope of the straight line by the reciprocal of the mass of the pycnometer contents at zero protein concentration (buffer or water). This procedure uses the relationship

$$(1 - \bar{V}\rho) = \frac{1 - w_2}{m} \frac{dm}{dw_2}$$

When the slope dm/dw_2 is evaluated at zero protein concentration, w_2 equals zero, and m equals the mass of water or buffer in the pycnometer. The partial specific volume, $\bar{V}$, is calculated from $(1 - \bar{V}\rho)$ by taking ρ as the density of the solvent—the solution used to obtain the zero concentration point. The straight line relating m and w_2 was calculated by the method of least squares, considering the zero concentration point to have no variation.

G. CALCULATION OF MOLECULAR WEIGHT BY THE SVEDBERG EQUATION

The Svedberg equation

$$M = \frac{RTS^0_{20,B}}{D^0_{20,B}(1 - \bar{V}\rho)}$$

was used to calculate the anhydrous molecular weights of the protein from the sedimentation and diffusion coefficients. In this equation, $S^0_{20,B}$ and $D^0_{20,B}$ represent the sedimentation and diffusion coefficients, respectively, of the protein at infinite dilution in the buffer, B, at 20 C. The buoyancy factor, $(1 - \bar{V}\rho)$, has the value corresponding to the same buffer medium (and at the same temperature) employed in the determinations of S and D.

H. EQUILIBRIUM SEDIMENTATION

Equilibrium sedimentation analyses were performed according to the meniscus depletion method of Yphantis[11] in 0.05 M sodium phosphate buffer at pH 6.8 and 0.1 ionic strength sodium Veronal buffer at pH 8.6; the protein had been dialyzed three times against the suspending buffer. The runs were done in 12-mm thick, Yphantis, six-channel, Kel-F centerpieces at protein concentrations ranging between 0.18 and 0.60 mg/ml. The third dialysate was used as the reference buffer in each case. The Spinco Model E

analytical ultracentrifuge used in these studies was equipped with an electronic speed control. Concentration distributions were recorded with the Rayleigh optical system, utilizing a symmetrical, medium slit-width lens mask. Rotor speeds were 29,500 rpm for phosphate buffer and 30,000 for Veronal buffer. The temperature of the runs was 20.0 C. Kodak Metallographic plates or 11-G plates were used for photographic recording. Sapphire windows were employed to minimize distortion of the interference pattern. A photograph taken after 16 hours of centrifugation was measured for the run in phosphate; photographs taken at 19 hours and at 22 hours were measured for the Veronal run.

The photographs of the fringe patterns were measured on a Nikon Shadowgraph Model 6C comparator, by both the fringe-location and the fringe-trace methods. In the fringe-location method, the fringe position is determined by measuring radial coordinates at half-fringe increments, as fringes are crossed in the radial traversal of the fringe pattern from the protein-depleted solvent region through the protein solution region. In the fringe-trace method, the vertical displacement of a single fringe from its meniscus position, or the average displacement of several fringes, is measured at a number of radial positions as the fringe is traversed from the region of protein-depleted solvent through the protein solution region. In most cases, when the fringe-trace method was used, three blank fringes in the central region of the diffraction envelope were measured at each radial position. The fringe distortion correction had no significant effect because the maximum deviation observed in blank runs was 1/10 fringe, which in these experiments amounts to about 30 μ. Because the uncertainty in the measurement of the fringes is of the order of 10 μ, the correction for blank shift was considered to be within reasonable experimental error. Moreover, the application of the correction did not significantly alter the values of the slopes obtained for $d(\ln c)/dr^2$.

In the fringe-trace method, the fringe displacement was measured at radial increments ranging from 500 to 50 μ on the photographic plate, depending on the vertical displacement; the interval was selected to give approximately equal intervals in vertical displacement. Measurements were made to the point where the fringes became blurred at the bottom and it was impossible to measure their positions with precision. Fringe displacements below 100 μ on the photographic plate were not included in the statistical analyses because of the large variation in measurement.[11]

The fringe displacement is proportional to refractive index increment (solution to solvent), which is proportional to protein concentration in dilute solutions. Thus the fringe displacement is proportional to protein concentration, and the concentration distribution at equilibrium in the centrifuge cell can be obtained from the fringe shifts at a number of levels in the cell.

Molecular weights are calculated with the formula[12]

$$M = \frac{2RT}{\omega^2(1 - \bar{V}\rho)} \frac{d(\ln c)}{dr^2}$$

In practice, ln (10F) or ln (100Y) was plotted versus r^2, where F is the number of intercepted fringes at half-fringe increments and Y is displacement (as measured on the photographic plate) of a fringe relative to the solvent base line in the protein-depleted region of the cell. The slopes $d\ln(10F)/dr^2$ and $d\ln(100Y)/dr^2$ are equivalent to $d(\ln c)/dr^2$, where c is protein concentration and r is distance of the radial position in the cell from the axis of rotation. $\bar{V}$ is the partial specific volume of the protein as determined in the solvent used in the equilibrium experiment; ρ is solvent density, ω is angular velocity of the rotor in radians per second, R is the gas constant, and T is the absolute temperature.

Slopes of the straight lines obtained in the plots of the natural logarithm of fringe displacement (10F or 100Y) versus square of radial position were evaluated by the method of least squares. Molecular weights and their standard deviations (as evaluated from standard deviations of slopes) were calculated for each set of measurements.

I. OSMOMETRY

Osmotic pressure measurements were performed in the Mechrolab High-Speed Membrane Osmometer, using Schleicher and Schuell Co. Bac-T-Flex Membrane Filters. Type B-19 membranes were used for osmometry with 0.05 M, pH 6.8 sodium phosphate buffer as the medium. Type B-20 membranes with smaller pore size (0.005μ) were used for osmometry with 0.1 ionic strength, pH 8.6 sodium Veronal as the medium. The third dialysate buffer, which was used in all the measurements, was de-aerated at room temperature. The membranes, which had been stored in alcohol, were rinsed, soaked and de-aerated in this dialysate buffer and then mounted on the osmometer chamber. Under the conditions of the measurements, at least 3 hours were required for equilibration of the membranes against third dialysate buffer. Solutions, in which the protein concentrations (determined by OD measurements) ranged between 1 and 11 mg/ml in the dialysate buffer, were introduced into the osmometer, and the membranes were allowed to equilibrate at least 10 minutes. The equilibrium osmotic pressures were read in centimeters of dialysate buffer. All measurements were made at 20.0 C. Several experiments were performed with phosphate buffer as the medium, using a different membrane in each experiment, and two different experiments were performed with Veronal buffer as the medium.

There was evidence for leaky membranes in the experiments in phosphate buffer, using B-19 membranes. Therefore, the osmometry results in phosphate will not be considered, except to state that molecular weight values in the neighborhood of 30,000 to 40,000 daltons were obtained.

The fact that horizontal straight lines represent the data in the osmometry experiments when π/c is plotted versus c (Fig. 4) shows that the solutions were ideal and made possible the calculation of M from the simple van't Hoff relationship

$$M = \frac{RT}{\pi/c}$$

M is molecular weight of the protein, R is the gas constant, T is the absolute temperature, and π/c is specific osmotic pressure in centimeters of Veronal buffer per gram per liter of protein. To calculate M as just described, it is necessary to convert the gas constant, R, from units of liter-atmospheres per mole per degree (Kelvin) to units of liter-cm buffer per mole per degree as follows:

$$\frac{\text{liter-atm}}{\text{mole-degree}} \times \frac{76.0 \text{ cm Hg}}{\text{atm}} \times \frac{13.55 \text{ g}}{\text{cmHg} \cdot \text{cm}^2} \times \frac{1}{\rho_B}$$

where ρ_B is density of the buffer employed. For the experiments in Veronal buffer, $\rho_B = 1.0033$ g/ml. Thus for the experiments in Veronal buffer at 20.0 C, the molecular weight was calculated from the formula

$$M = \frac{24688.4 \frac{\text{(liter-cm buffer)}}{\text{mole}}}{\pi/c}$$

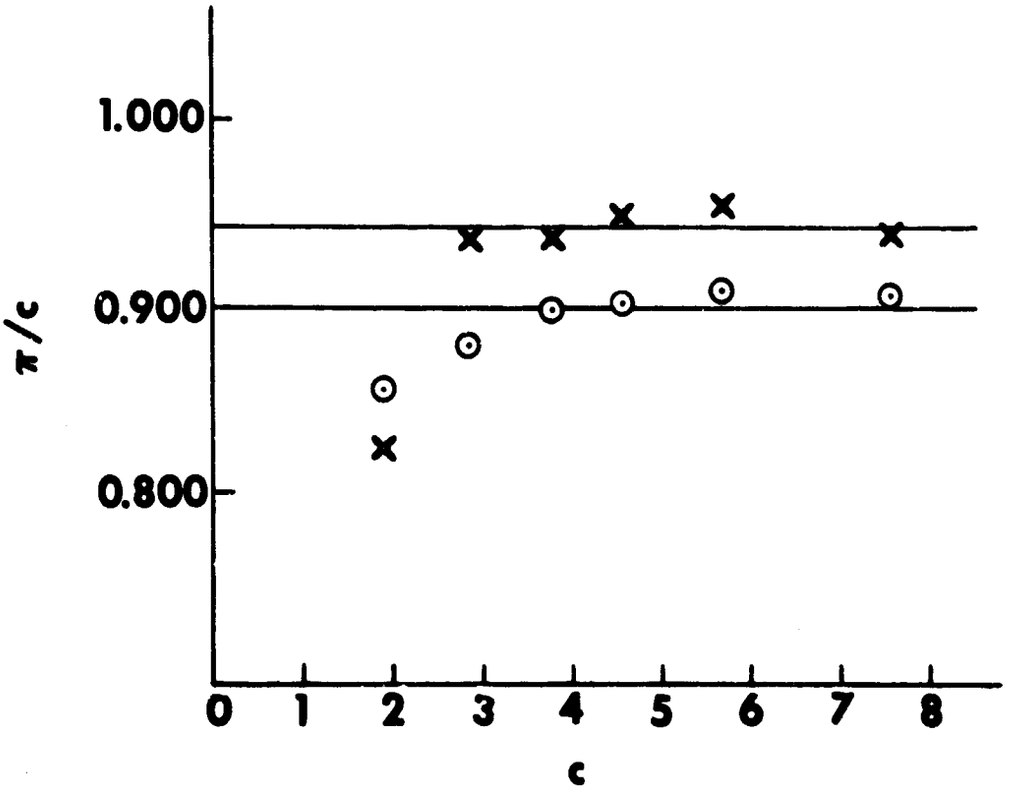

Figure 4. Plots of Specific Osmotic Pressure (π/c) versus c for Two Osmometry Experiments on Staphylococcal Enterotoxin B Suspended in 0.1 Ionic Strength, pH 8.6 Veronal Buffer. The horizontal, straight, least squares lines were drawn through the five points representing the final measurements made after equilibration of the membranes. Units: π in centimeters Veronal buffer, c in grams per liter.

III. RESULTS

Only one component was observed in the sedimentation, diffusion, and electrophoresis experiments. Typical schlieren patterns obtained in electrophoresis and sedimentation experiments are shown in Figures 5 and 6, respectively.

Measurement of the maximum ordinate of the schlieren pattern obtained following 7 hours under free-boundary electrophoresis conditions in Veronal buffer indicated zero displacement and thus zero electrophoretic mobility at pH 8.6. A measurement on the photograph obtained following 8 hours under free-boundary electrophoresis conditions in borate buffer indicated a possible slight displacement, from which an electrophoretic mobility of about 3×10^{-6} cm^2/volt-sec was calculated. This displacement was considered to be insignificant.

Plots of natural logarithm of displacement of the boundary from the axis of rotation versus time of sedimentation of the protein were linear in all cases. A typical plot is shown in Figure 7.

Sedimentation coefficients plotted as functions of concentration of enterotoxin B protein are shown graphically in Figure 8 for runs in phosphate and Veronal buffers. The sedimentation results for both lot C-40 and lot C-41 are represented. The linear least squares intercepts, corresponding to sedimentation coefficients at infinite dilution, and their standard deviations are shown in Table 1.

Figure 9 is a set of photographs of toxin protein diffusing in phosphate buffer in the centrifuge cell. The quantity $u^2 (1 - \omega^2 St)$, representing the squared spread (corrected for rotor speed) of the concentration distribution between the 0.25 and 0.75 c_o levels in the boundary, is plotted as a function of time of diffusion in phosphate buffer in Figure 10. The diffusion coefficient and its standard deviation, calculated from the least squares slope of this plot, are $8.42 \pm 0.25 \times 10^{-7}$ cm^2/sec. For the diffusion experiment in borate buffer, the calculations yielded $7.96 \pm 0.34 \times 10^{-7}$ cm^2/sec for the diffusion coefficient and its standard deviation. In the calculations of molecular weight, these values for the diffusion coefficient were considered to be equal to the infinite dilution values because the initial concentrations of 1.2 mg/ml were quite low.

The densities of enterotoxin B protein solutions are plotted as functions of concentration of protein in Figure 1 for the experiment in distilled water, in Figure 2 for the experiment in Veronal buffer, and in Figure 3 for the experiment in phosphate buffer in which the dialysate was used for dilution and comparison. The values $(1 - \bar{V}\rho)$ and their standard deviations, calculated from the linear least squares slopes, are presented in Table 2. The corresponding values for $\bar{V}$ are also shown.

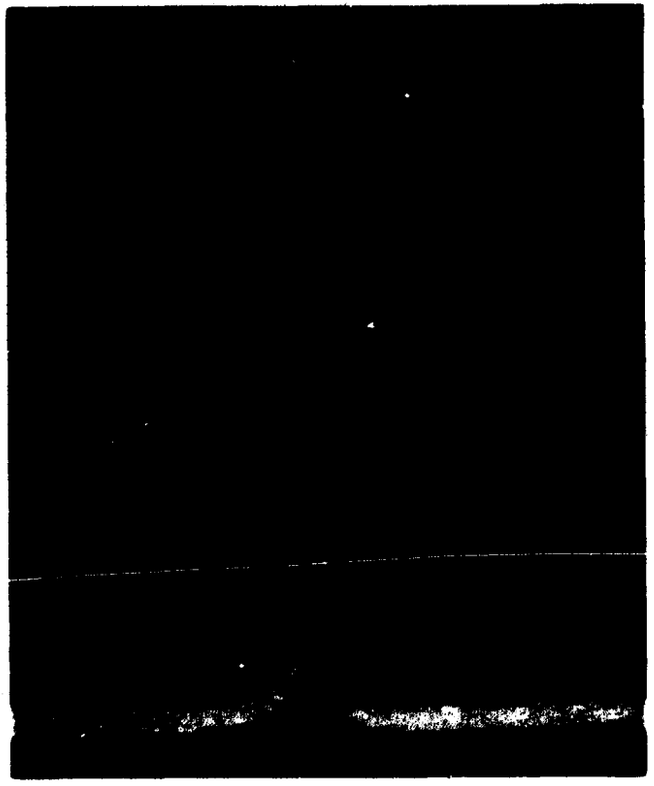

Figure 5. Photograph of Schlieren Pattern from Staphylococcal Enterotoxin B Protein in 0.1 Ionic Strength Veronal, pH 8.6. The photograph was taken in the Perkin-Elmer Model 238 electrophoresis apparatus, using an analyzer angle of 45°, after the protein had been subjected to a current of 4 milliamps for 2.4 hours following boundary formation and sharpening.

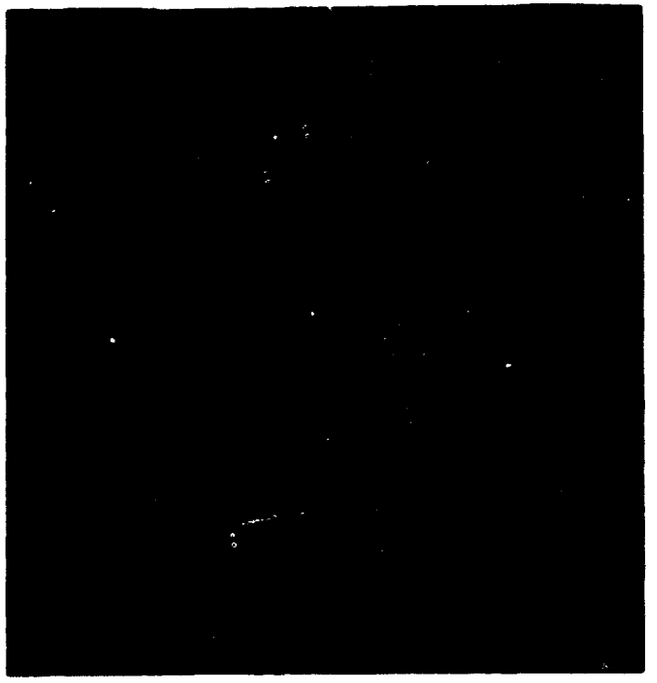

Figure 6. Photograph of Schlieren Pattern Corresponding to Staphylococcal Enterotoxin B Protein at a Concentration of 7.15 mg/ml in 0.1 Ionic Strength Veronal Buffer, pH 8.6. The exposure was made at an analyzer angle of 60°, 43 minutes after reaching speed of 59,780 rpm in a Spinco Model E analytical centrifuge. Sedimentation from left to right.

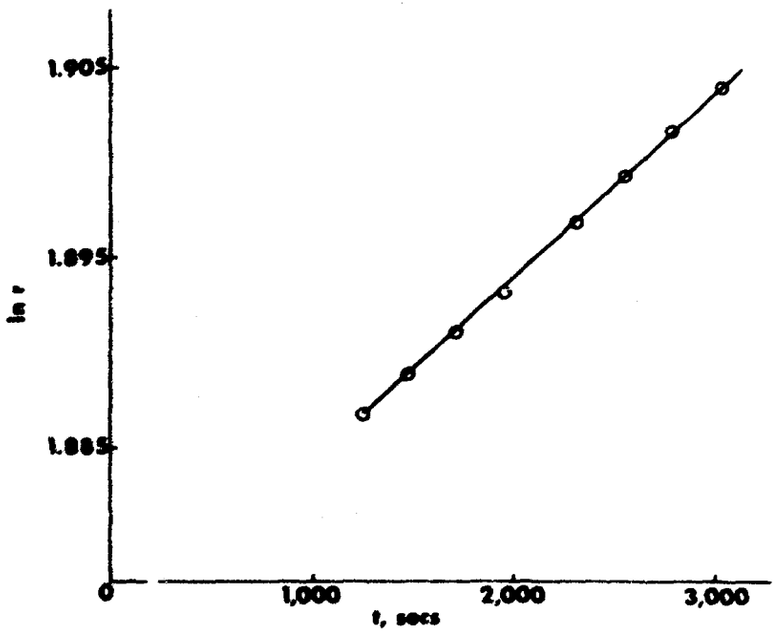

Figure 7. Sedimentation, at 20 C, of Staphylococcal Enterotoxin B at a Concentration of 7.15 mg/ml in 0.1 Ionic Strength Veronal, pH 8.6. The ordinate is the natural logarithm of the distance in centimeters from the axis of rotation of the maximum ordinate of the schlieren pattern, and the abscissa is the time after reaching speed of 59,780 rpm in the Spinco Model E analytical centrifuge. The calculated sedimentation coefficient is 2.47 x 10^{-13} seconds.

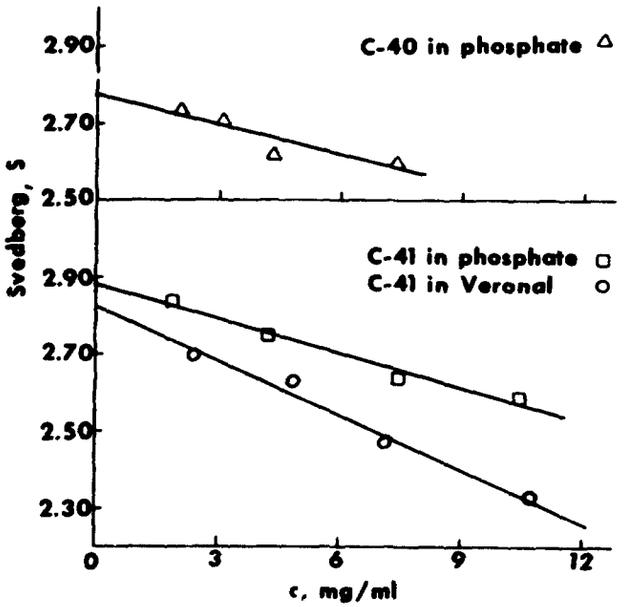

Figure 8. Sedimentation Coefficients, in Svedbergs (10^{-13} seconds), at 20.0 C, as a function of Concentration of Two Lots of Staphylococcal Enterotoxin B in the Indicated Buffers. In each case, the least squares straight line representing the data has been extrapolated to infinite dilution.

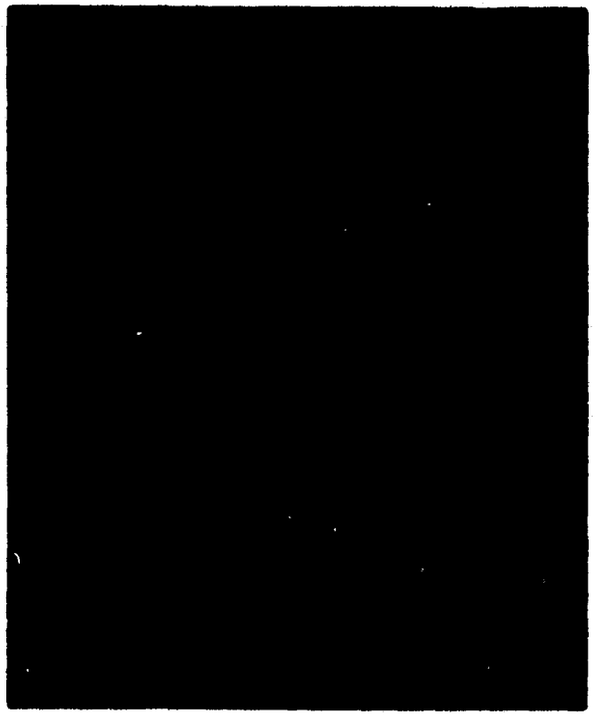

Figure 9. Diffusion, at 4 C, of Staphylococcal Enterotoxin B Protein at a Concentration of 1.2 mg/ml in 0.05 M Phosphate Buffer, pH 6.8. The ultraviolet optical system exposures were made at 8-minute intervals, beginning 1,080 seconds after boundary formation in the Spinco Model E analytical centrifuge, which was operated at a speed of 21,740 rpm.

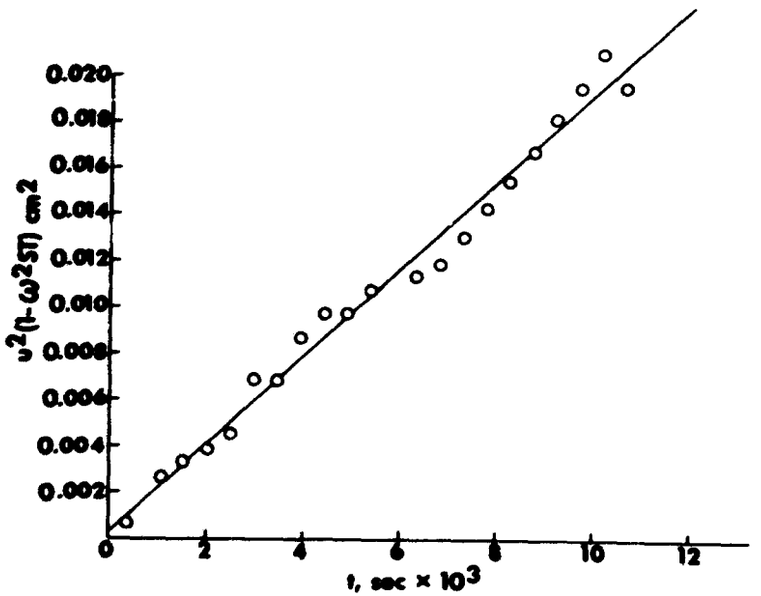

Figure 10. Diffusion of Staphylococcal Enterotoxin B at a Concentration of 1.2 mg/ml in 0.05 M Phosphate Buffer, pH 6.8. The diffusion run was made in the Spinco Model E analytical untracentrifuge at 21,740 rpm and 4 C. The ordinate represents values of the squared distances between the 25% and 75% concentration levels, as corrected for rotor speed and to 20 C.

TABLE 1. SEDIMENTATION COEFFICIENTS OF STAPHYLOCOCCAL ENTEROTOXIN B AT INFINITE DILUTION IN DIFFERENT MEDIA

Lot No.	Medium[a]	$S^0_{20,B}$[b], 10^{-13} sec
C-41	Phosphate	2.88±0.02
C-41	Veronal	2.83±0.03
C-40	Phosphate	2.78±0.04

a. 0.05 M sodium phosphate at pH 6.8 and 0.1 ionic strength sodium Veronal at pH 8.6.
b. The values represent the least squares intercepts and their standard deviations, derived from plots of sedimentation coefficients versus concentrations of enterotoxin protein. $S^0_{20,B}$ stands for sedimentation coefficient at infinite dilution in buffer, B, at 20 C.

TABLE 2. BUOYANCY FACTORS AND PARTIAL SPECIFIC VOLUMES OF STAPHYLOCOCCAL ENTEROTOXIN B PROTEIN IN DIFFERENT MEDIA AT 20.0 C

Medium[a]	$(1 - \bar{V}\rho)$[b]	ρ, g/ml	$\bar{V}$, ml/g
Phosphate	0.2624±0.0039	1.0045	0.734
Veronal	0.2623±0.0009	1.0033	0.735
Distilled Water	0.2813±0.0009	0.99823	0.720

a. 0.1 ionic strength sodium Veronal at pH 8.6 and 0.5 M sodium phosphate at pH 6.8.
b. The values were calculated from least squares slopes of plots of densities versus concentrations of enterotoxin protein; the standard deviations listed were calculated from standard deviations of the least squares slopes.

A value of 31,800±1,000 daltons was calculated for the anhydrous molecular weight of the protein, using the values of the sedimentation and diffusion coefficients and the buoyancy factor for protein in phosphate buffer. From the sedimentation coefficient and buoyancy factor in Veronal and the diffusion coefficient in borate, a value of 33,000±1,400 daltons was calculated for the protein at the isoelectric point, pH 8.6. The standard deviations of these molecular weights were derived from the standard deviations of the sedimentation and diffusion coefficients and of the buoyancy factors, as applied in the Svedberg equation. A summary of the experimental parameters used to calculate molecular weights with the Svedberg equation is given in Table 3; molecular weight results, as well as comparative values from Wagman et al.[1] and Bergdoll et al.,[2] are also given there.

Figure 11 is a photograph of the interference fringe patterns obtained in the experiment in Veronal buffer. Straight lines were obtained in all the plots of the natural logarithm of fringe displacement as a function of radius squared in the sedimentation equilibrium experiments at positions corresponding to fringe displacements greater than 100 μ. A typical plot is shown in Figure 12. This plot represents data obtained by measurement of fringe displacements corresponding to sedimentation equilibrium of the protein, following centrifugation for 19 hours in Veronal buffer; the initial concentration of protein was 7/12 mg/ml.

The molecular weights calculated from data obtained through a number of independent measurements of the Rayleigh interference patterns for Veronal and phosphate buffer media are summarized in Table 4. The third solution in the run in phosphate was not included in the results because a shift of less than one fringe was observed at the concentration employed (~2/11 mg/ml), leading to unreliable data. The standard deviations of the molecular weights are derived from the standard deviations of the corresponding buoyancy factors ($1 - \bar{V}\rho$) and from standard deviations of slopes of least squares plots of ln fringe displacement versus r^2.

Molecular weights of 27,400±400 daltons and 26,200±200 daltons were calculated from the osmotic pressure data for the experiments in 0.1 ionic strength Veronal buffer, pH 8.6. The osmotic pressure data for the two experiments are shown in Figure 4. The ratio π/c is plotted as a function of c; π is the osmotic pressure in centimeters of Veronal buffer as read on the osmometer, and c is concentration of protein in grams per liter. The points corresponding to the lowest concentrations used in each experiment were omitted in the calculations of molecular weight because the least concentrated solutions were the first introduced into the osmometer in each experiment and the osmometer was not considered to be equilibrated until the second solution was introduced. A molecular weight and its standard deviation of 26,800±300 daltons were calculated from the ten selected points pooled from the two experiments.

TABLE 3. COMPARISON OF PARAMETERS USED TO CALCULATE MOLECULAR WEIGHT BY THE SVEDBERG EQUATION

	Veronal		Phosphate	
Parameter	Bergdoll et al.	Trkula et al.	Wagman et al.	Trkula et al.
S^0_{20} [a], sec	2.78×10^{-13}	$2.83 \pm 0.03 \times 10^{-13}$	2.89×10^{-13}	$2.88 \pm 0.02 \times 10^{-13}$
D^0_{20} [a], cm^2/sec	8.22×10^{-7}	$7.96 \pm 0.34 \times 10^{-7}$ [b]	7.72×10^{-7}	$8.42 \pm 0.24 \times 10^{-7}$
$(1 - \bar{V}\rho)_{20}$	0.2753	0.2623±0.0009	0.2583 [c]	0.2624±0.0039 0.2813±0.0009 [c]
$\bar{V}$, ml/g	0.726 [d]	0.735	0.743 [c]	0.734 0.720 [c]
M [e]	30,777 [f]	33,000±1,400	35,300	31,800±1,000

a. Our values for S and D are for buffer solvent; the other values are corrected to water as solvent.
b. This value for D is for borate buffer, pH 8.6, as solvent.
c. These values are for distilled water as solvent, pH not recorded.
d. Calculated from amino acid composition of the protein.
e. Calculated by the Svedberg equation.
f. This value is questionable, because a value of 0.2753 is calculated for the buoyancy factor for distilled water as solvent and $\bar{V}$ = 0.726 ml/g; this yields a molecular weight value of 30,000 daltons.

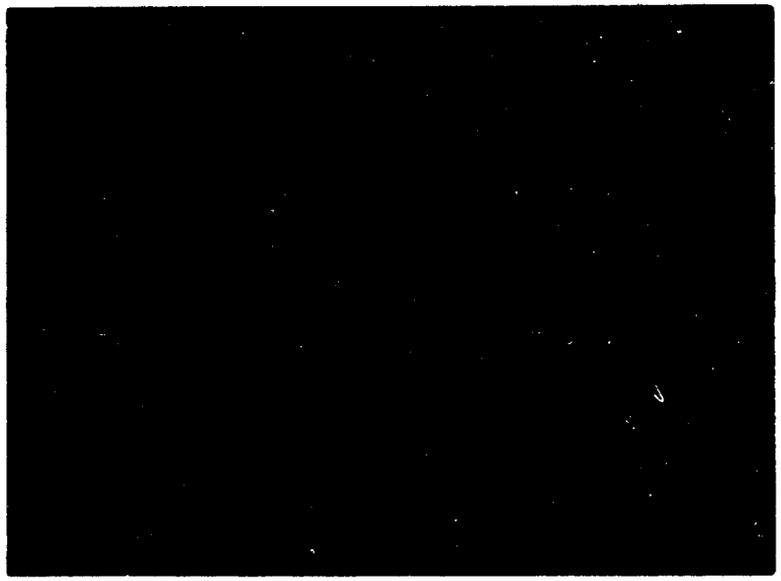

Figure 11. Sedimentation Equilibrium of Staphylococcal Enterotoxin B. Rayleigh fringe pattern from Yphantis six-compartment cell. Protein concentrations were 7/12, 5/12, 1/4 mg/ml in 0.1 ionic strength Veronal buffer, pH 8.6. Exposure was for 15 seconds on a Kodak II-G plate, after 19 hours centrifugation at 30,000 rpm and 20.0 C in a Spinco Model E analytical centrifuge. Sedimentation from left to right.

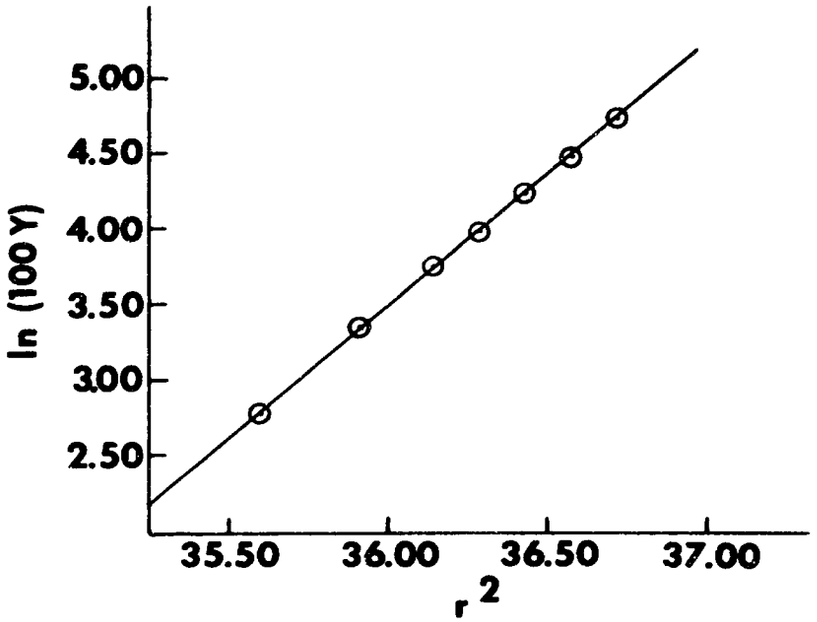

Figure 12. Plot of Log_e Fringe Displacements (100 Times the Fringe Displacement in Millimeters on the Photographic Plate) versus Square of Radial Position (in Centimeters) During Sedimentation Equilibrium of Staphylococcal Enterotoxin B. The toxin protein was run at a concentration of 7/12 mg/ml in 0.1 ionic strength Veronal buffer for 19 hours at a speed of 30,000 rpm and at 20 C. The molecular weight of the protein, calculated from the slope of the straight line, is 32,700 ± 200 daltons.

TABLE 4. MOLECULAR WEIGHTS OF STAPHYLOCOCCAL ENTEROTOXIN B PROTEIN DETERMINED BY THE YPHANTIS HIGH-SPEED EQUILIBRIUM METHOD

Analysis Number[a]	Molecular Weight, daltons	Standard Deviation	Method[b]	Number Fringes Measured	Concentration,[c] mg/ml	Time of Centrifugation at Speed, hours
			Determination in 0.05 M Phosphate Buffer, pH 6.8; 29,500 rpm, 20 C			
1a	28,700	500	L	8	6/11	16
1b	28,400	400	T	1	6/11	16
2a	32,700	500	T	5	4/11	16
2b	32,800	500	T	1	4/11	16
2c	31,600	500	L	1	4/11	16
Average:	30,400±2,000					
			Determination in 0.1 Ionic Strength Veronal Buffer, pH 8.6; 30,000 rpm, 20 C			
3a	31,000	100	L	3	7/12	19
3b	33,400	500	T	3	7/12	19
3c	32,700	200	T	3	7/12	19
4a	29,800	200	L	3	5/12	19
4b	29,800	300	T	3	5/12	19
5a	29,600	500	L	3	3/12	19
5b	29,800	200	T	3	3/12	19
6	32,500	200	L	3	7/12	22
7	30,300	300	L	3	5/12	22
8	31,400	300	L	3	3/12	22
Average:	31,000±1,300					

a. The fringe measurement corresponding to a particular concentration of solution for a particular time of centrifugation is designated as an analysis. The letter designations refer to independent measurements of the same fringe pattern (corresponding to a particular concentration of protein and a particular time of centrifugation) either by a different method (fringe-location or fringe-trace) or simply a repeat measurement at a different time.

b. L stands for measurements by the fringe-location method and T stands for measurements by the fringe-trace method.

c. The concentrations are listed as fractions to reflect the method of loading the cell compartments; for example, the concentration 7/12 mg/ml was obtained by adding 0.05 ml of Veronal buffer to 0.07 ml of protein at a concentration of 1 mg/ml and 0.02 ml of Spinco FC-43 liquid fluorocarbon oil to yield a total volume of 0.14 ml, but a protein solution volume of 0.12 ml; in the phosphate run 0.03 ml of FC-43 was deposited in each compartment, thus leaving 0.11 ml for the solution.

IV. DISCUSSION

The fact that there was either no, or at least no significant, displacement of the toxin under the electrophoresis conditions in either Veronal or borate buffer indicates that the toxin protein is isoelectric in Veronal and borate buffers, 0.1 ionic strength at pH 8.6. The presence of a single, sharp schlieren peak during prolonged electrophoresis in each buffer is evidence for the electrical homogeneity of the protein.

Values of the experimental parameters used by us, by Bergdoll et al.,[3] and by Wagman et al.[1] for calculation of M by the Svedberg equation are shown for comparison in Table 3.

The infinite dilution value of the sedimentation coefficient at 20.0 C (S^0_{20}) seems to be somewhat less for lot C-40 (2.78 Svedbergs) than for lot C-41 (2.88 Svedbergs) toxin protein in 0.05 M phosphate buffer, pH 6.8. The value of S^0_{20} for lot C-41 toxin in 0.1 ionic strength Veronal, pH 8.6, is about the same (2.83 Svedbergs) as the value in phosphate. These values of S^0_{20} are not significantly different from the value obtained by Bergdoll et al. of 2.78 Svedbergs for $S^0_{20,w}$ derived from velocity sedimentation runs in 0.1 ionic strength Veronal, pH 8.6, and the value of 2.89 Svedbergs obtained by Wagman et al. for $S^0_{20,w}$ derived from experiments in 0.05 M phosphate buffer, pH 6.8. Thus, there is no apparent difference in S^0_{20} among the various lots of toxin employed, or between S^0_{20} in phosphate and in Veronal.

The apparent greater dependence of S on c in Veronal compared with phosphate shown in Figure 8 is not considered significant in view of the fact that Wagman et al. show a greater dependence of S on c in pH 6.8 phosphate than do Bergdoll et al. for experiments in pH 8.6 Veronal.

Because there is no significant difference between the S^0_{20} values we obtained for sedimentation in Veronal (zero net charge on the protein) and pH 6.8 phosphate buffers, it can be concluded that the salt concentration of the buffer is sufficient to eliminate charge effects.

The diffusion coefficients in phosphate (8.42×10^{-7} cm^2/sec) and borate (7.96×10^{-7} cm^2/sec) buffers are not considered to be significantly different from each other nor from the value of 8.22×10^{-7} cm^2/sec obtained by Bergdoll et al. for diffusion of the toxin in 0.1 ionic strength, pH 8.6 Veronal buffer in agar gel. The value of 7.72×10^{-7} cm^2/sec obtained by Wagman et al. for diffusion in 0.05 M phosphate buffer, pH 6.8, differs considerably from our value of 8.42×10^{-7} cm^2/sec in phosphate buffer; but this difference is still considered to be within the range of experimental error. The fact that the diffusion coefficients are the same in phosphate and Veronal indicates that there is no charge effect for the diffusion in 0.05 M phosphate buffer.

The values 0.2623 and 0.2624 derived for buoyancy factors for the protein at 20 C in Veronal and phosphate, respectively, are considered to agree with each other within experimental error. The value 0.2813 derived for the buoyancy factor of the protein at 20 C in distilled water is probably significantly different from the other two.

When the buoyancy factors, $(1 - \bar{V}\rho)$, are converted to partial specific volumes, $\bar{V}$, the values 0.735 and 0.734 ml/g for $\bar{V}$ in Veronal and phosphate, respectively, do not differ significantly from each other (Table 2). Neither do they differ significantly from the values of 0.731 and 0.726 ml/g calculated by Spero* and by Bergdoll et al., respectively, from the amino acid composition of the protein. Also, the value 0.743 ml/g in water obtained by Wagman et al. agrees within experimental error[13] with our values in Veronal and phosphate.

There does not seem to be a pH effect on $\bar{V}$, observed by Charlwood,[14] according to which $\bar{V}$ increases considerably as the pH is increased or decreased from the isoelectric pH. The lack of a pH effect on $\bar{V}$ is consistent with the constancy of S between Veronal and phosphate media. Charlwood and Ens[15] noted a decrease in S for bovine serum albumin at pH values less than the isoelectric point. The fact that the values we obtained for $\bar{V}$ in Veronal and phosphate, 0.735 and 0.734 ml/g, respectively, are almost the same indicates that there are no significant Donnan effects in the phosphate buffer in which the protein is not isoelectric. This agrees with the results obtained by Casassa and Eisenberg.[8] Also it is interesting to note that the value ($\bar{V}$ = 0.727 ml/g) in the determination in phosphate in which dialyzed protein was diluted and compared with stock phosphate buffer does not differ significantly from the value ($\bar{V}$ = 0.734 ml/g) obtained in the experiment in which dialyzed protein was diluted with dialysate and the dialysate was used in obtaining the comparison density.

Our value for $\bar{V}$ of 0.720 ml/g at 20 C in distilled water is considered to differ significantly from our values in phosphate and Veronal. We do not know how to explain this result. The value ($\bar{V}$ = 0.693 ml/g) obtained in an experiment in which protein was dialyzed against distilled water, then made 0.05 M in phosphate, and then diluted and compared with 0.05 M phosphate buffer, pH 6.8, at 20 C is considered unreliable, because there was more scatter of the points about the regression line than in any of the other experiments.

A value ($\bar{V}$ = 0.716 ml/g) obtained for the protein at 25 C in phosphate by dilution of the dialyzed protein with dialysate and comparison with dialysate also is probably significantly different from $\bar{V}$ = 0.734 ml/g for the protein at 20 C in phosphate. We cannot explain the lesser value for $\bar{V}$ at 25 C than at 20 C, in view of the fact that Kay[9] reported a positive temperature dependence of $\bar{V}$ for several muscle proteins.

* Spero, L., 1968, personal communication.

The agreement between our M values (as calculated by the Svedberg equation) in Veronal and phosphate confirms that there is no significant charge effect and no significantly different ion binding effects in the two solvents. The results just discussed and the data in Table 3 show that the discrepancies between the molecular weights we calculated from the Svedberg equation and those calculated by Bergdoll et al. and by Wagman et al. can be explained in terms of experimental errors. The value in Veronal of 30,777 reported by Bergdoll et al. is lower than our value of 33,000 because of nonsignificant differences in S^0_{20}, D^0_{20}, and $\bar{V}$; the value in phosphate of 35,300 reported by Wagman et al. is greater than our value of 31,800, mainly because of the nonsignificant difference in D^0_{20}. Thus, we conclude that the four values for M derived by the method of sedimentation and diffusion agree within experimental error.

The straight lines obtained in all the lnc versus r^2 plots are consistent with the interpretation that the protein solutions are monodisperse and ideal. If the lines were concave upwards, heterogeneity would be indicated; if concave downwards, non-ideality would be indicated.[18] Straight lines were obtained in these plots at all the different loading concentrations over the same range of fringe displacements. This is also consistent with molecular homogeneity of the protein.[11]

We do not consider our values for M obtained by the Yphantis high-speed equilibrium method to be significantly different from each other in the phosphate series or the Veronal series. Values are listed in Table 4 for different loading concentrations of toxin protein, different times of centrifugation at the equilibrium speed, and different methods of measurement. The mean value for the experiment in phosphate buffer is 30,400±2,000 daltons; the mean value for the experiment in Veronal buffer is 31,000±1,300 daltons.

It should be pointed out that Wagman et al. obtained higher values (34,400 average) for M of the toxin protein in phosphate by the Archibald approach-to-equilibrium method than we did by the Yphantis method (30,400 average). Because this difference cannot be attributed to the $(1 - \bar{V}\rho)$ factor, the results of measurements using the Archibald method as compared with the Yphantis method appear to differ.

Again, because the values for molecular weight by the sedimentation equilibrium method in phosphate and Veronal agree so well, there is no evidence for charge effect in phosphate buffer or for significantly different ion-binding effects in phosphate as compared with Veronal buffers. The agreement between molecular weight values at the several loading concentrations employed is evidence that the solutions are essentially thermodynamically ideal at these concentrations.

The agreement between the molecular weight values obtained by the methods of sedimentation equilibrium and sedimentation and diffusion can be taken as further confirmation of homogeneity.

The horizontal straight lines obtained in plotting π/c versus c in the osmometry experiments are consistent with solution ideality over the range of concentrations employed (no dependence of π/c on c). This agrees with the criteria of linear lnc versus r^2 plots and equal molecular weights at several loading concentrations in the equilibrium experiments.

The mean value of molecular weight for the toxin protein in Veronal obtained by osmometry in two experiments is 26,800±300 daltons. This value is lower than the mean values obtained for the toxin by the methods of sedimentation and diffusion and sedimentation equilibrium. The lower molecular weight of the toxin by osmometry might be explained either on the basis of experimental error or on the basis of heterogeneity of the protein. However, the rather considerable heterogeneity required for the discrepancy between M_w and M_n ought to be detectable by sedimentation and sedimentation equilibrium analysis. Therefore, it seems more reasonable to attribute the difference to experimental error.

It is not possible to estimate, from our experimental data, the effects of ion binding on the molecular weight of the protein. It has been determined that about six phosphate groups are bound by a molecule of the toxin protein (exhaustively dialyzed against distilled water, from 0.03 M phosphate buffer at pH 6.5 to 6.7).* This would add about 1,000 daltons to the molecular weight of the toxin protein, under the conditions of our experiments. This would not contribute significantly to the molecular weight. Because the molecular weight of the protein in Veronal is the same, within experimental error, as in phosphate, no significant difference is expected in the binding of the barbiturate and the phosphate ions. It is possible to test for significant binding by determining S in KCl, for example, where little binding is expected.**

The average value for molecular weight of the toxin protein in Veronal buffer, 31,000±1,300 daltons, obtained by the sedimentation equilibrium method, is considered to be the most reliable value we obtained. This is because the method does not involve as many measurements, and is thus not subject to as many sources of inaccuracy, as the method of sedimentation and diffusion and because it is not as sensitive as osmometry to possible low molecular weight components. Furthermore, the value in Veronal is for an isoelectric protein, obviating the necessity for any corrections due to charge effects.

This value for the molecular weight of 31,000±1,300 daltons is consistent, within experimental error, with all our other determinations and with the values obtained by Wagman et al. and Bergdoll et al. Moreover, it is consistent with the value of 29,600 obtained by viscometry of reduced and alkylated toxin in 6 M guanidine.***

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* Spero, L., 1968, personal communication.
** Saroff, H.A., 1968, personal communication.
*** Spero, L., 1968, personal communication.

Evidence for the homogeneity of the toxin protein used in this investigation is the single, Gaussian schlieren diagrams obtained in both electrophoresis and sedimentation analyses and the straight lines obtained in the lnc versus r^2 plots in the sedimentation equilibrium experiments. Moreover, straight lines were obtained in these plots for different loading concentrations, two in the phosphate experiment and three in the Veronal experiment, over the same range of fringe displacements. This latter evidence for homogeneity is discussed by Yphantis.[11] The agreement between the molecular weight values obtained by sedimentation equilibrium and by sedimentation and diffusion is also evidence for homogeneity. We cannot discount the possibility of some molecular heterogeneity of the protein, in view of the discrepancy between the osmometry values and the values obtained by sedimentation methods. But we believe most of the discrepancy is due to experimental error because the large degree of heterogeneity otherwise required should have been detectable by the various experimental methods used.

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13. ABSTRACT

The molecular weight of staphylococcal enterotoxin B was determined independently and by methods not previously applied to the protein; the results agree well with those obtained in two earlier studies. In order to check the previous determinations, analyses were performed in 0.05 M sodium phosphate buffer, pH 6.8, and in 0.1 ionic strength sodium Veronal buffer, pH 8.6, the isoelectric point of the protein. The sedimentation and diffusion method yielded 33,000±1,400 daltons in Veronal buffer and 31,800±1,000 daltons in phosphate buffer. The Yphantis meniscus-depletion sedimentation equilibrium method yielded average values of 31,000±1,300 daltons in Veronal and 30,400±2,000 daltons in phosphate. Osmometry yielded an average value of 26,800±300 daltons in Veronal buffer.

14. Key Words

Staphylococcal enterotoxin B
Molecular weight
Sedimentation
Diffusion
Sodium phosphate
Sodium Veronal
Buffers

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