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THE SUBUNIT COMPOSITION OF *ESCHERICHIA COLI* ALKALINE PHOSPHATASE IN 1 M TRIS

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SUMMARY

1. The sedimentation coefficient of *Escherichia coli* alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) was determined at very low concentrations by the methods of active enzyme sedimentation in the analytical ultracentrifuge and zonal Tris gradient sedimentation in the preparative ultracentrifuge.

2. The value of $s^{\circ}_{20,w}$ for active enzyme species is the same in 1.0 M NaCl, 0.15 M KBr, and 1.0 M Tris, pH 8.0. This value, 6.04 S, is nearly identical to that obtained by measurement at much higher protein concentrations followed by extrapolation, 6.22 S.

3. The enzyme species corresponds to a dimer over the concentration range of less than 0.017 mg/ml to 0.57 mg/ml as determined by sedimentation velocity, both in 1 M Tris and in the other solvents studied. This conclusion is independent of the presence or absence of substrate.

INTRODUCTION

The subunit structure of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) in 1 M Tris is a matter of some concern since Tris markedly increases the rate of utilization of substrates[1,2]. This effect has been associated with the distinct ability of Tris to serve as a phosphate acceptor in a transphosphorylation reaction. Efforts have been made to deduce a mechanism which would account for the effect of Tris based upon the assumption that Tris serves as a kinetic acceptor[3,4]. It has been tacitly assumed that the molecular structure of alkaline phosphatase is not drastically altered in 1 M Tris and, in particular, the possible separation of the dimer into monomeric subunits has not been entertained because of the prevailing belief that monomeric units are not enzymically active.

A recent paper has placed all this in doubt. It has been suggested that alkaline phosphatase at low concentration in 1 M Tris dissociates into monomers that are more active than the dimeric species[5]. Using frontal gel chromatography in 1 M Tris it was found that at protein concentrations exceeding 0.4 μg/ml the enzyme behaves as

expected for the dimer, $8.6 \cdot 10^5$ daltons, but at lower protein concentrations the elution volume increases. These findings are highly significant for understanding of alkaline phosphatase and for interpretation of the kinetic effects of high concentrations of Tris.

The dissociation of the enzyme in 1 M Tris places the effect of Tris in a new perspective, since kinetic studies are made at low enzyme concentrations where the enzyme would be completely dissociated. Moreover, the kinetic data presented by WINZOR[5] suggest that monomeric and dimeric species both exhibit enzymic activity and that the monomer is more active than the dimer. This conclusion is contrary to present thought and merits further investigation. In view of these circumstances it is imperative to obtain further evidence on this question of the dissociation of the alkaline phosphatase dimer.

WINZOR[5] points out that the identification of the dimer is made from ultracentrifugation studies at high protein concentration. We have decided to examine this matter by ultracentrifugation, but by using methods of ultracentrifugation that do not require high protein concentrations. These methods are zonal velocity sedimentation in the preparative ultracentrifuge and active enzyme sedimentation[6] in the analytical ultracentrifuge. The first method is well known, but the latter method has not yet received wide application.

We have employed the technique of active enzyme sedimentation to measure the sedimentation coefficient of alkaline phosphatase by direct determination of the rate of advance of the product front produced by the movement of a band of enzyme through a sedimentation solvent containing substrate. These experiments are conducted by layering a few μl of enzyme solution onto the top of a column of sedimentation solvent with the aid of a Type I band-forming centerpiece[7]. As the enzyme moves through the solvent, the *p*-nitrophenyl phosphate substrate, which does not absorb at the wavelength selected, is converted to absorbing product, *p*-nitrophenol. This technique may be employed with highly impure enzyme preparations, since only the active species is detected. The low enzyme concentrations used, a few μg/ml, or less, ensure the absence of concentration dependence.

Active enzyme sedimentation has been previously used to measure the sedimentation coefficient of glutamate dehydrogenase by the more complex method of measuring the rate of advance of the peak corresponding to the difference in product concentrations between succeeding scans[6]. We find that, for our system, the rate of advance of the midpoint of the product front itself, a much simpler measurement, yields essentially the same result.

We find that the sedimentation coefficient of alkaline phosphatase is the same using 1.0 M NaCl, 0.15 M KBr or 1.0 M Tris, pH 8.0, as sedimentation solvents. The average value of $s^\circ_{20,w}$ determined by the active enzyme sedimentation method, 6.04 ± 0.08 S, is nearly identical to that obtained from boundary sedimentation of alkaline phosphatase and extrapolation to infinite dilution. We therefore conclude that the active enzyme species corresponds to the dimer.

EXPERIMENTAL

Chemicals

p-Nitrophenyl phosphate hexahydrate was obtained from the Aldrich Chemical

Co. Tris, reagent grade trizma base, and bovine serum albumin were obtained from the Sigma Chemical Co.

Enzyme preparation

The enzyme was prepared from *Escherichia coli* by the osmotic shock technique of NEU AND HEPPEL[8] and purified by chromatography on DEAE-cellulose using a NaCl gradient[9]. Enzyme prepared in this manner was found to have an activity comparable to the crystalline preparation of MALAMY AND HORECKER[10]. The final preparations were stored in 0.01 M Tris at pH 8.0 at 4°. Enzyme samples stored in this way retained 95 to 100% of their original activity after 3 months.

Zonal sedimentation in a Tris gradient

Sedimentation experiments were performed using a SW 50 L rotor in a Model L2 65 B Beckman Spinco ultracentrifuge for 10 h at 45 500 rev./min and 25°.

Using a Pasteur pipet with a hooked tip, 70 μl of enzyme solution in 0.01 M Tris at pH 8.0 was carefully layered on a preformed gradient of 0.90 to 1.10 M Tris at pH 8.0. High concentrations (0.57 mg/ml) and low concentrations (0.017 μg/ml) of alkaline phosphatase were used with bovine serum albumin as a marker (20 mg/ml). The enzyme solutions become diluted about 20-fold at zone center during the sedimentation. At the end of the run 35 ± 1 fractions of 10 drops each were collected. Fractions from the high concentration of alkaline phosphatase were assayed by introducing a 0.05-ml sample into a cuvette containing 10^{-3} M *p*-nitrophenyl phosphate in 3 ml of 1 M Tris at 25°. The change in absorbance at 400 nm was then monitored. Fractions from low alkaline phosphatase concentrations were assayed by pipetting 3 ml of assay media directly into each fraction and incubating at room temperature for 88 h. The bovine serum albumin was determined by the protein method of LOWRY *et al.*[11] using the full fraction in a final volume of 2.3 ml. The absorbance at 750 nm was 3.30 absorbance units/mg. Each centrifuge tube contained 4.9 ml and the column height was 4.3 cm. Duplicate determinations defined the peak position to within ± 10 drops (± 1 fraction), or ± 0.12 cm.

Viscosity and density measurements

The density and viscosity of 1.0 M Tris, pH 8.0, were determined at 20.0° and 25.0°. Viscosity determinations were made using an Ostwald viscometer immersed in a constant temperature bath. The viscosity, η_{Tr}, of 1.0 M Tris, pH 8.0, was calculated according to the equation

$$\frac{\eta_{Tr}}{\eta_w} = \frac{t_{Tr}}{t_w}\frac{\varrho_{Tr}}{\varrho_w} \qquad (1)$$

where t_{Tr} and t_w are the times for a fixed volume of 1.0 M Tris and water to fall through the capillary and ϱ_{Tr} and ϱ_w are the densities of the liquids. All viscosity measurements were performed in triplicate, and drainage times were reproducible to $\pm 0.5\%$. Densities were determined with a 50-ml pycnometer standardized with water. The following values were obtained in 1.0 M Tris, pH 8.0: $\varrho_{20} = 1.0411$ g/ml; $\varrho_{25} = 1.0389$ g/ml; $\eta_{20} = 1.405$ cp; $\eta_{25} = 1.291$ cp.

Analytical centrifugation

Active enzyme and boundary sedimentation experiments were carried out in the Beckman Model E analytical ultracentrifuge equipped with photoelectric scanner, multiplex accessory and electronic speed control. All experiments were performed at 20° and 56 000 rev./min in a clear An D rotor. Double sector, charcoal-filled epon, 12 mm centerpieces were used for the boundary sedimentation.

Band sedimentation of the enzyme as detected by the rate of advance of the product front was carried out with Type 1 double sector charcoal-filled epon 12 mm centerpieces[7], except as otherwise noted. The volume of sedimentation solvent was 0.35 ml.

Sedimentation coefficients were calculated by a least-squares fit to a straight line plot of log (r_m) *vs.* t, where r_m is the location of the half maximum product concentration. Standard deviations are expressed as 95% confidence levels using Student's t distribution. Correction to $s_{20,w}$ was made by the Svedberg method, using the calculated value of 0.73 ml/g for the partial specific volume of alkaline phosphatase.

RESULTS

Boundary sedimentation of alkaline phosphatase

Alkaline phosphatase was found to sediment as a single boundary in 0.15 M KBr, 0.01 M Tris, pH 8.0, and 20°. A slight concentration dependence was observed,

TABLE I

BOUNDARY SEDIMENTATION OF *E. coli* ALKALINE PHOSPHATASE

The solvent is KBr, 0.15 M; Tris, 0.01 M, pH 8.0, 20°. Scans were recorded at 280 nm.

Enzyme concentration (mg/ml)	$s_{20,w}$ *value*
1.25	6.12 ± 0.03
0.46	6.21 ± 0.05
0.062	6.27 ± 0.30

Table I, and values of $s_{20,w}$ were obtained which are in substantial agreement with those reported by others[12]. The value of $s_{20,w}$ obtained at the lowest protein concentration used in these experiments is approximately equal to the value at infinite dilution, $s^{\circ}_{20,w}$.

Band sedimentation of alkaline phosphatase monitored by product front advance

The results of experiments in which a band of alkaline phosphatase was sedimented through a variety of solvents containing substrate, *p*-nitrophenyl phosphate, are presented in Table II. The amount of protein in each case was below the limit of optical detection and the progress of the band of enzyme was accordingly monitored by recording the concentration profile of product formed, *p*-nitrophenol, at regular time intervals. The rigorous calculation of the sedimentation coefficient for this reacting system requires that account be taken of both the movement of the enzyme and of the sedimentation and diffusion of the product[6]. In practice the latter two factors

TABLE II

BAND SEDIMENTATION OF THE ACTIVE ALKALINE PHOSPHATASE–*p*-NITROPHENYL PHOSPHATE COMPLEX

All experiments were conducted at 20°, pH 8.0, and scans were recorded at 400 nm.

Solvent	*p-Nitrophenyl phosphate*	*Lamella vol. (μl)*	*Initial enzyme concn. (ng/ml)*	$s^\circ_{20,w}$
KBr, 0.15 M; Tris, 0.01 M	$2 \cdot 10^{-4}$	20	33	6.16 ± 0.09
NaCl, 1.0 M; Tris, 0.01 M	$2 \cdot 10^{-4}$	10	67	6.09 ± 0.08
		20	37	6.20 ± 0.11
Tris, 1.0 M, pH 8.0	$2 \cdot 10^{-4}$	6	450	6.14 ± 0.05
	$2 \cdot 10^{-4}$	10	67	5.91 ± 0.08
	$2 \cdot 10^{-4}$	20	37	6.07 ± 0.03
	$1 \cdot 10^{-3}$	40*	18	5.92 ± 0.15
	$2 \cdot 10^{-2}$	20	67	5.84 ± 0.08

* Centerpiece was 30 mm charcoal-filled epon, type Band I, double sector.

may be neglected and, with the aid of some further approximations, it has been shown that the sedimentation coefficient of the principal enzyme species may be obtained by following the rate of movement of the maximum in the difference curve between succeeding scans[6]. Alternatively, the sedimentation coefficient may be calculated from the slope of a plot of the logarithm of the position of the inflection point of the advancing product front as a function of time[13]. The inflection point is often satis-

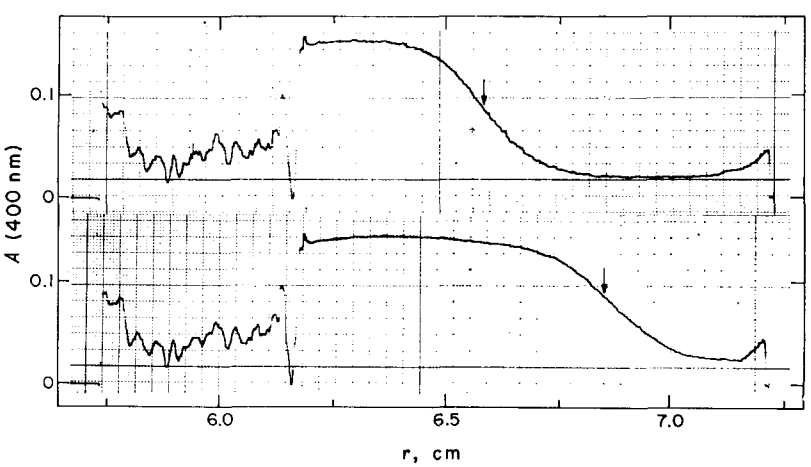

Fig. 1. Photoelectric scanner records of the product front profiles resulting from the sedimentation of alkaline phosphatase through 1.0 M Tris, pH 8.0, $2.0 \cdot 10^{-4}$ M *p*-nitrophenyl phosphate. The absorbance of product, *p*-nitrophenol, is indicated in the ordinate scale, and the abscissa represents the distance from the center of rotation. The run conditions were 56 000 rev./min, 20°, 1.2 cm charcoal-filled epon Type I band-forming centerpiece. The arrows represent the location of the half-maximum concentration upper trace, 62.5 min and lower trace, 111.5 min after arriving at speed. The sample solution formed an initial lamellum of 10 μl, and the sedimentation solvent was 0.35 ml.

factorily approximated by the location of the midpoint of the product front. Substantially the same results were obtained using either method, and correspondingly we routinely employed the much simpler midpoint location determination. Fig. 1 illustrates the product front profiles at 62.5 min and 111.5 min after attaining full speed (56 000 rev./ min) with an initial lamellum of 10 μl and other conditions as noted in the legend. The arrows indicate the locations of r_m, the position of half maximum concentration.

The data summarized in Table II reveal that, within experimental error, the sedimentation coefficient of the active alkaline phosphatase–*p*-nitrophenyl phosphate complex is the same in 0.15 M KBr, 1.0 M NaCl and 1.0 M Tris, pH 8.0. The results further indicate a lack of dependence of $s^\circ_{20,w}$ upon lamella volume, initial enzyme concentration, total amount of enzyme, or concentration of substrate above $2 \cdot 10^{-4}$ M.

The average value of $s^\circ_{20,w}$ calculated from the data of Table II is 6.04 $\pm$ 0.08 S, a value which is slightly lower than the estimated value of 6.27 $\pm$ 0.25 S from the boundary sedimentation results of Table I. The reason for this small difference is not known, but it might possibly reflect a change in the frictional coefficient of the principal enzyme species in these experiments as compared to that of the protein as monitored by boundary sedimentation. The absence of any large change in $s^\circ_{20,w}$ in any of these three solvents as compared to the boundary sedimentation results clearly indicates, however, that no gross conformational change, such as dissociation into subunits, occurs.

The enzyme activity as determined in the analytical ultracentrifuge

The total amount of product formed at any time can be evaluated from the

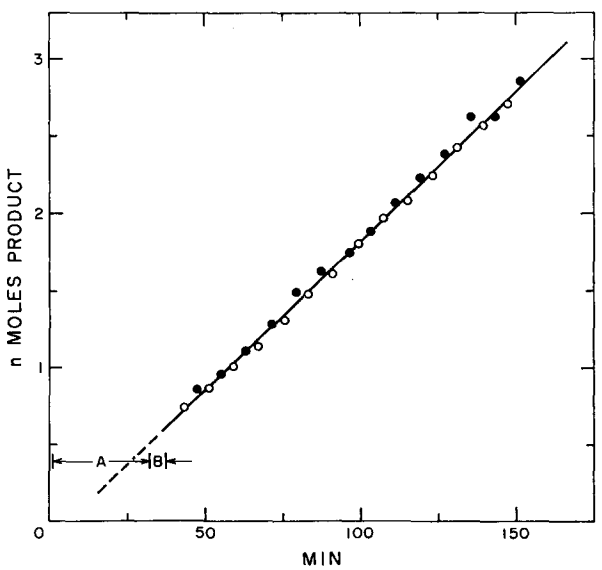

Fig. 2. Rate of production of product, *p*-nitrophenol, during active enzyme sedimentation. The ordinate represents the amount of product as calculated with Eqn. 2 for initial lamellum volumes of 20 μl (○) and 10 μl (●). Region A indicates the time elapsed during which the rotor speed was maintained at 12 000 rev./min; Region B indicates the time required to accelerate to the final operating speed of 56 000 rev./min. Other centrifugation conditions are listed under Fig. 1.

absorbance profile of product throughout the analytical cell. The mass of product in μmoles is given by the expression

$$m = \frac{200 f \varphi \Delta r}{\varepsilon d} \Sigma p_i r_i \qquad (2)$$

where φ, the sector angle, is equal to 0.04363 radian; Δr is the constant summation interval in cm; d is the first scanner absorbance calibration distance in cm; ε is the molar extinction coefficient of *p*-nitrophenol at 400 nm, $1.7 \cdot 10^4$; r_i is the radial coordinate in cm, and p_i is the pen excursion in cm measured from the same ordinate location as d. The centrifuge calibration factor f is the ratio of absorbance as determined in the Gilford Model 2000 Spectrophotometer to that determined in the ultracentrifuge. We determined a value $f = 1.53$ at 400 nm, over the region between 0.0 and 0.2 absorbance unit.

The total amount of product produced during the sedimentation process was found to increase linearly with time regardless of the volume of the lamellum containing the enzyme at the start of the experiment, as shown in Fig. 2. The amount of enzyme activity of the sedimenting enzyme in 1.0 M Tris, pH 8.0, 20°, was determined from the slope of this plot to be 43 μmoles/min per mg enzyme, as compared to the value of 53 determined by direct measurement outside the centrifuge.

The results of Fig. 2 also indicate that the details of the enzyme concentration distribution throughout the cell do not significantly influence the calculation of the sedimentation coefficient. Thus, the initial enzyme concentration in the experiment beginning with the 20-μl lamellum is half the initial concentration of the enzyme in the experiment beginning with the 10-μl lamellum.

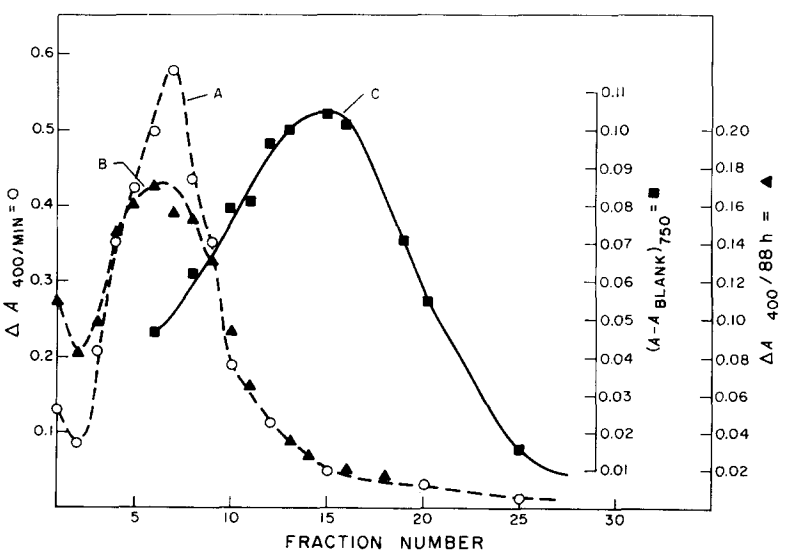

Fig. 3. Sedimentation profile for alkaline phosphatase and bovine serum albumin in 0.9 to 1.1 M Tris gradient centrifugation: Curve A, alkaline phosphatase layered at high concentration; Curve B, alkaline phosphatase layered at low concentration (see text); Curve C, bovine serum albumin layered at 20 mg/ml. The left hand side of the graph refers to the bottom of the centrifuge tube.

Zonal Tris gradient sedimentation

The sedimentation velocity of alkaline phosphatase was the same whether very high enzyme concentrations or very low enzyme concentrations were used (Fig. 3). In both cases alkaline phosphatase was found to sediment more rapidly than bovine serum albumin, molecular weight 70 000. Had the alkaline phosphatase dissociated into monomers, it would have been found higher in the tube than the bovine serum albumin marker. The sedimentation coefficient of alkaline phosphatase was calculated using bovine serum albumin as a reference.

It is shown in APPENDIX that, in the shallow density and viscosity gradients used in this work, the difference in sedimentation coefficient between alkaline phosphatase and the bovine serum albumin marker $s_{20,w} = 4.315$ is

$$\Delta s = \frac{1}{\omega^2 t} \ln (r_2/r_1) \tag{3}$$

where ω is the rotor speed in rad/sec, t is the time, and r_2 and r_1 are the distances of the enzyme and of bovine serum albumin from the center of rotation.

Our value for Δs was 1.33 S, which corrected to standard conditions gives a value for $\Delta s_{20,w}$ of 1.91 S. The value of $s_{20,w}$ for alkaline phosphatase is 1.91 + 4.31 = 6.22 S. This value is in close agreement with the previously reported value for the dimer and with our other measurements.

DISCUSSION

The results of active enzyme sedimentation experiments indicate that alkaline phosphatase sediments as a dimer in 1 M Tris (pH 8.0, 20°). The enzyme activity was uniformly found to sediment as a single component at a velocity corresponding to a dimer, even though the enzyme was reported to be monomeric in frontal gel chromatography at comparable and higher enzyme concentrations[5].

The analytical ultracentrifuge and chromatographic techniques differ in that the substrate is present in active enzyme sedimentation but is absent in frontal gel chromatography. It is therefore possible that dissociation of the enzyme is prevented by the substrate. This might happen if the substrate were bound preferentially to the dimer. We investigated this possibility using zonal Tris gradient sedimentation in the absence of substrate with bovine serum albumin present as a marker. We were able to detect no enzyme activity at positions in the gradient other than that corresponding to dimeric alkaline phosphatase, over a concentration range from 0.017 μg/ml to 0.57 mg/ml. We were therefore unable to confirm the existence of an alkaline phosphatase monomer by sedimentation velocity.

The present application of active enzyme sedimentation was facilitated by the use of a photoelectric scanner and multiplex accessory, permitting the quantitative determinations of enzyme specific activities with multiple samples in the same rotor. The alternative absorption photographic system would be adequate for sedimentation coefficient measurements, but a monochromator and high intensity light source are in general required.

The substrate concentrations used in our experiments were selected to be greatly in excess of the Michaelis constant, $\leqslant 10^{-5}$ M, so that the sedimenting species was not the free enzyme but rather the enzyme–substrate complex and about 5%

phosphoryl–enzyme intermediate. The enzyme itself is diluted greatly during the experiment due to both diffusion and to radial dilution. Using the exact solution of RUBIN AND KATCHALSKY[14] for the ultracentrifuge differential equation under these conditions, we calculate that alkaline phosphatase is diluted to a final concentration equal to 10% of the initial value over a 1.0-cm sedimentation path at 56 000 rev./min. Because of the exceedingly low initial enzyme concentrations used, this further dilution during the experiment was without effect on the measured values of the sedimentation coefficient.

Our conclusion is that alkaline phosphatase does not dissociate in 1 M Tris.

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APPENDIX

The linear density and viscosity gradients which pertain in these experiments give rise, to first order, to a linear variation in sedimentation coefficient with radial position

$$s(r) = s + s'(r - r_0) \tag{A1}$$

where s is the value at the top of the gradient, r_0, and $s' = \mathrm{d}s/\mathrm{d}r$. The differential equation then becomes

$$\frac{\mathrm{d}r}{\mathrm{d}t} = s\omega^2 r + s'\omega^2 r(r - r_0) \tag{A2}$$

and has its solution

$$(1 - kr_0)\, s\omega^2 t = \ln(r/r_0) - \ln[1 + k(r - r_0)] \tag{A3}$$

where $k = s'/s$. Since $k(r - r_0) \ll 1$, Eqn. A3 may be approximated by

$$s\omega^2 t = \frac{\ln(r/r_0) - k(r - r_0)}{1 - k r_0} \tag{A4}$$

Eqn. A4 is written for two components and the difference is taken, assuming that k is the same for both. The result for the difference in sedimentation coefficients is

$$s\omega^2 t = \frac{\ln(r_1/r_2) - k\Delta r}{1 - k r_0} \tag{A5}$$

The value of k may be estimated with the aid of the Svedberg correction and the assumption of linear density and viscosity gradients.

$$\eta = \eta_0 + \eta'(r - r_0) \tag{A6}$$

$$\varrho = \varrho_0 + \varrho'(r - r_0) \tag{A7}$$

Combination of Eqns. A1, A6 and A7 with the Svedberg correction leads to the result

$$k = -\left(\frac{\eta'}{\eta_0} + \frac{\varrho'}{1 - \varrho_0}\right) \tag{A8}$$

where all terms of higher powers of $(r - r_0)$ have been neglected. With the aid of the values of η and ϱ for 1.0 M Tris, and the assumption of linearity, it is estimated that $\eta'/\eta_0 = 0.0047$, $\varrho'/\varrho_0 = 0.0055$, and hence $k = 0.0010$.

Typical values of r_0, r^0 and r_1 in experiments with the SW 50 L rotor are 4.70 cm, 6.5 cm and 7.7 cm, respectively. In this case the value of $\ln(r_1/r_2)$ is 0.170, whereas the righthand side of Eqn. A5 yields the correct value 0.166. Hence, to within 2% the approximate relationship given by Eqn. A9 may be used in place of Eqn. A5,

$$\Delta s = \frac{1}{\omega^2 t} \ln(r_2/r_1) \tag{A9}$$

REFERENCES

1 D. J. PLOCKE AND B. L. VALLEE, *Biochemistry*, 1 (1962) 1039.
2 A. GAREN AND C. LEVINTHAL, *Biochim. Biophys. Acta*, 38 (1960) 470.
3 J. DAYAN AND I. B. WILSON, *Biochim. Biophys. Acta*, 81 (1964) 620.
4 T. REID AND I. B. WILSON, *Biochemistry*, 10 (1971) 380.
5 D. J. WINZOR, *Biochim. Biophys. Acta*, 200 (1970) 423.
6 R. COHEN, B. GIRAND AND A. MESSIAH, *Biopolymers*, 5 (1967) 203.
7 J. VINOGRAD, R. RADLOFF AND R. BRUNER, *Biopolymers*, 3 (1965) 481.
8 H. NEU AND L. HEPPEL, *J. Biol. Chem.*, 240 (1965) 3685.
9 R. SIMPSON, B. VALLEE AND G. TAIT, *Biochemistry*, 7 (1968) 4336.
10 M. MALAMY AND L. HORECKER, *Biochemistry*, 3 (1964) 1889.
11 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
12 M. L. APPLEBURY AND J. E. COLEMAN, *J. Biol. Chem.*, 244 (1969) 308.
13 W. R. BAUER, in preparation.
14 M. RUBIN AND A. KATCHALSKY, *Biopolymers*, 4 (1966) 579.