Biochimica et Biophysica Acta, 327 (1973) 176–185

BBA 67048

THE USE OF ABSORPTION OPTICS TO MEASURE DISSOCIATION OF YEAST ENOLASE INTO ENZYMATICALLY ACTIVE MONOMERS

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(Received June 4th, 1973)

SUMMARY

The possibility that the monomers of yeast enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11), which normally exists as a dimer, have enzymatic activity has been examined. Dissociation of the dimer, as measured by sedimentation velocity, does not occur in the presence of 0.001 M $MgCl_2$ at 25 °C in the concentration range of 3 to 200 μg/ml. However, at 40 °C enolase dissociates with 50% dissociation occuring at 40 μg/ml. The dissociation at 40 °C was confirmed by determining molecular weights from sedimentation equilibrium experiments. These molecular weights were determined by modifying the Archibald equation for use with the photoelectric scanner. Accurate molecular weights can easily be determined in 2 h with a very minimum of calculations. Use of the reacting enzyme sedimentation technique not only shows that yeast enolase dissociates in the presence of the substrate, 2-phosphoglycerate, but also that the monomer retains all of its original catalytic activity. In this technique the sedimentation of a band of enolase through its reaction mixture is followed by observing the appearance of the product phosphoenolpyruvate. This appearance is measured at 240 nm using the photoelectric scanner attachment to an analytical ultracentrifuge. In the presence of 2.44 mM 2-phosphoglycerate, yeast enolase is 50% dissociated at 0.2 μg/ml. Sedimentation coefficients were measured by following the migration of the plateau half-height as in the measurement of conventional sedimentation coefficients. The sedimentation coefficients were in excellent agreement with those obtained by conventional means. Also included in this paper is a log–log graph of sedimentation coefficients *versus* molecular weights using values derived from the literature.

INTRODUCTION

The glycolytic enzyme enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) isolated from yeast cells, exists as a dimer, *in vitro*, according to Brewer and Weber[1]. These authors concluded that yeast enolase, when dissociated at low protein concentrations (50% dissociated at 20 μg/ml), consisted of monomers devoid of enzymatic activity. Dissociation of the enolase dimer can also be accomplished

with 1 M KCl or KBr, also resulting in enzymatically inactive monomers (Brewer and Weber[2]). Evidence reported by Keresztes-Nagy and Orman[3] using gel filtration techniques suggest, however, that yeast enolase exists as a monomer at 43 °C and is enzymatically active in the monomeric form. The results reported here, where actual measurements of sedimentation coefficients were performed on the reactive species as it is reacting, also show that enolase monomers are active. This sedimentation analysis was performed by observing the appearance of product, phosphoenolpyruvate, which is formed as a band of enolase sediments through the reaction mixture ($MgCl_2$ and 2-phosphoglyceric acid).

Warburg and Christian[4] showed that yeast enolase, which they crystallized as the mercury salt, had a mol. wt of 67 000. Other work by Malmström *et al.*[5], Brewer and Weber[1], Gawronski and Westhead[6] and by Brewer *et al.*[7] confirmed these results. However, Mann *et al.*[8] reported a mol. wt of 88 000 for the native particle and 44 000 for the dissociated molecule, which are in agreement with the values reported for enolase isolated from other sources (Winstead and Wold[9], Ruth *et al.*[10]). Sedimentation velocity experiments and sedimentation equilibrium techniques, using a modification of the Archibald technique adapted for use with absorption optics, confirm the results of Mann *et al.*[8], that the molecular weight of enolase is approximately 90 000. These results have been reported in a preliminary form (Holleman[11]).

MATERIALS

Yeast enolase and 2-phosphoglyceric acid were purchased from Sigma Chemical Co. Tris (Schwarz Bioresearch) and sucrose (Mann) were ultrapure research grade reagents. All solutions were prepared with triple distilled water.

METHODS

Sedimentation

Analytical sedimentation data were obtained using a Spinco Model E ultracentrifuge equipped with a split-beam automatic photoelectric scanning optical system (Lamers *et al.*[12]). The sedimentation experiments were performed in 12- and 30-mm double sector cells. Enolase samples were prepared by dissolving the stock enzyme in buffer and diluting this stock to give the desired concentration assuming an extinction coefficient of $E_{280\ nm}$ equals 9.0 (Malmström[13], Westhead and McLain[14]).

Sedimentation equilibrium

Because of condensation of oil on the lenses, which occurs at the elevated temperature (40 °C) during the course of a normal sedimentation equilibrium experiment (24 h), it was necessary to obtain molecular weights in a very short time. This requirement was met by using the Archibald technique in conjunction with absorption optics as first described by de Groot *et al.*[15] and later by Holleman[16]. This method allows for calculation of molecular weights at very low concentrations with relatively little work. According to Archibald[17], the two variables needed in order to obtain molecular weights are the concentration (c) of the protein as well as its concentration gradient, dc/dr, at either the cell meniscus or cell bottom.

$$\frac{1}{r}\frac{dc}{dr} = \frac{M(1 - \bar{v}\varrho)\, c\, \Omega^2}{RT} \qquad (1)$$

As an ultracentrifuge equipped with a split-beam automatic photoelectric scanning optical system provides a direct measurement of concentration throughout the cell, it is possible to obtain concentration (c) directly at the cell bottom or cell meniscus. Also, the concentration gradient, dc/dr, can be obtained from the same output data, for the slope of the trace as it passes through the meniscus will be dc/dr. Fig. 1 is a schematic drawing of a typical scanner trace, including the line drawn through the meniscus, whose slope is equal to dc/dr and whose point of intersection with the meniscus is equal to concentration at the meniscus. All sedimentation equilibrium runs were performed at 17 000 rev./min with 1.0 ml of the enzyme dissolved in 0.1 M Tris–HCl, 1 mM $MgCl_2$, 5% sucrose, pH 7.4, in the sample side of a 30-mm double sector cell and 1.2 ml of buffer in the reference side. A partial specific

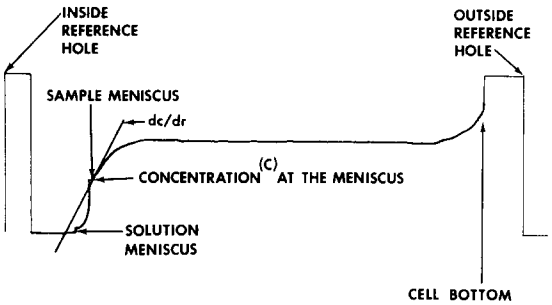

Fig. 1. Schematic drawing of an Archibald approach to equilibrium scanner trace to illustrate the means of obtaining the slope of the trace (dc/dr) as it passes through the meniscus and of calculating the point of intersection of the trace (c) with the meniscus.

volume of 0.735 cm^3/g for yeast enolase (Bergold[18]), was used in the molecular weight calculations. Scans were obtained at 280 nm and at 236 nm for low protein concentrations.

Sedimentation velocity

Sedimentation velocity studies were performed by following absorbance at either 280 or 236 nm, using a Spinco Model E analytical ultracentrifuge equipped with a split-beam photoelectric scanning system. All sedimentation coefficients were corrected to $s_{20,w}$ according to Schachman[19].

Sedimentation velocity analyses at low protein concentrations ($< 10\ \mu g/ml$) were carried out according to the active enzyme centrifugation method of Cohen *et al.*[20,21], using the absorption scanning optical system. This procedure involves layering a band of enzyme on top of its substrate and observing the formation of product as the band of enzyme sediments. Specifically 10 μl of enolase, dissolved in 0.1 M Tris–HCl, 1 mM $MgCl_2$, pH 7.4, is layered on top of 0.34 ml of the reaction mixture contained in one sector of a 12-mm charcoal-filled Epon Type 1 band forming centerpiece. The reaction mixture consists of the above buffer *plus* 2.44 mM 2-phosphoglyceric acid and 5% sucrose which provides a density difference between enzyme and substrate thus insuring proper layering of enzyme. The reference side of the cell contains 0.40 ml of the reaction mixture. The speed of centrifugation was

60 000 rev./min and the banding of the enzyme, which was observed in the schlieren optical system, occured between 1000 and 2000 rev./min.

$$\text{2-phosphoglycerate} \rightleftarrows \text{phosphoenolpyruvate} + H_2O \qquad (2)$$

As the reaction product phosphoenolpyruvate (see above) absorbs ultraviolet light at 240 nm, in contrast to the substrate 2-phosphoglycerate, the progress of the enzyme band through the assay mixture can be followed at 240 nm by recording scans at various time intervals. If during the course of the ultracentrifugation the amount of product formed is very small in relation to the substrate concentration (see Discussion), the half height of the plateau will correspond to the center of the enzyme band as it sediments through the reaction mixture. Consequently sedimentation coefficients were determined by following the half height of the plateau during sedimentation. Fig. 2 shows a series of traces obtained from a velocity run performed at 2 μg of enolase per ml. Using this method it was possible to measure sedimentation coefficients for enolase down to 0.07 μg/ml.

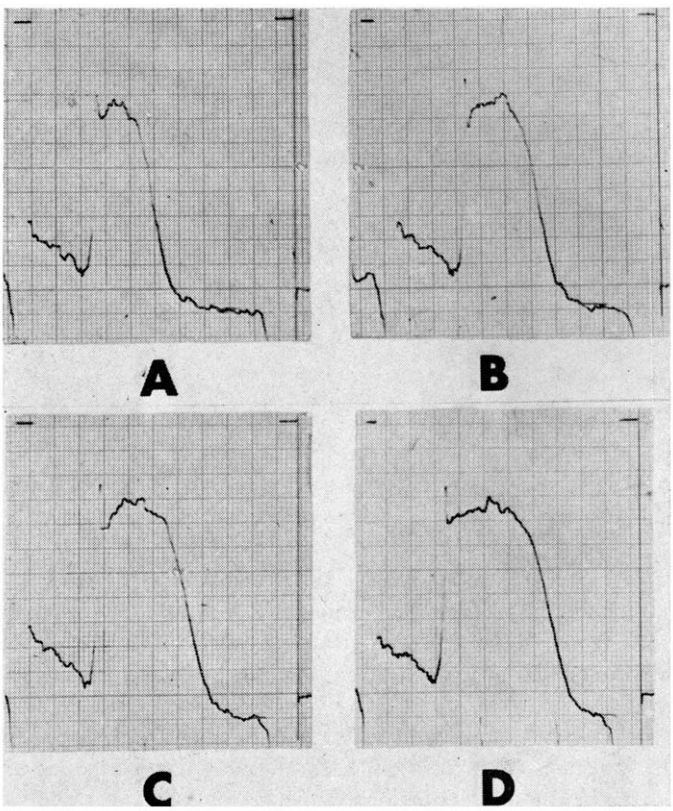

Fig. 2. Scanner traces taken at 8-min intervals during an active enzyme centrifugation of yeast enolase. 10 μl of the enzyme solution was layered onto 0.34 ml of 0.1 M Tris–HCl, 1.0 mM $MgCl_2$, 2.44 mM 2-phosphoglyceric acid, 5% sucrose, pH 7.4. Scanner traces were taken at 240 nm, 60 000 rev./min, 40 °C and at an enzyme concentration of 2 μg/ml. centrifugation is from left to right.

RESULTS

A series of sedimentation velocity experiments, performed at 25 and 40 °C, in the absence of the substrate 2-phosphoglycerate but in the presence of 1 mM $MgCl_2$, are shown in Fig. 3. No dissociation occurred at 25 °C (solid circles) the lowest concentration being 3 μg/ml. Extrapolation of the 25 °C data to zero protein concentration yielded a value for the sedimentation coefficient of native enolase of 5.85 S. This value is to be compared with the value of 5.9 S found by Malmström[13]. An entirely different situation exists, however, at 40 °C where dissociation begins to occur at 70 μg/ml with the degree of dissociation increasing with decreasing enzyme concentration. Extrapolation of the curve to zero protein concentration gives a sedi-

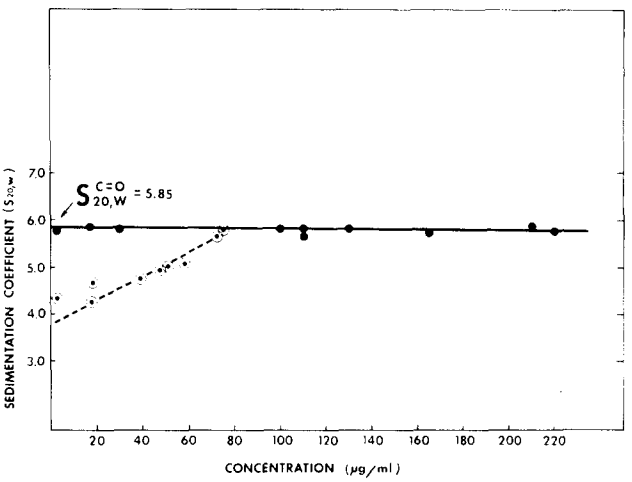

Fig. 3. Sedimentation coefficients of yeast enolase as a function of concentration and temperature ●, 25 °C; ○, 40 °C. Experiments were conducted in 0.1 M Tris–HCl, 1.0 mM $MgCl_2$, pH 7.4, at 60 000 rev./min.

mentation coefficient of 3.7 S for the enolase monomer. In contrast to the experiments conducted in the presence of 2-phosphoglycerate (see below), which included 5% sucrose in the buffer system, no sucrose was used in these experiments. Sedimentation velocity experiments in the presence of sucrose, however did not alter the results shown in Fig. 3.

Assuming a mol. wt of 90 000 (Mann *et al.*[8]) for the dimer and 45 000 for the monomer, a 50% dissociated solution of enolase will have an apparent weight average molecular weight of 67 500. Fig. 4 is a graphical representation of the sedimentation coefficients of various globular proteins as a function of their molecular weight, using values combed from the literature. The values, plotted on a log–log scale, give a straight line relationship from mol. wt 12 000 to 400 000. Using this curve it can be estimated that an enolase mixture composed of equal parts of monomer and dimer will have an approximate sedimentation coefficient of 4.8 S. Using this value in conjunction with the data shown in Fig. 3, it can be determined that at 40 °C, yeast enolase in the absence of 2-phosphoglycerate is 50% dissociated at 40 μg/ml.

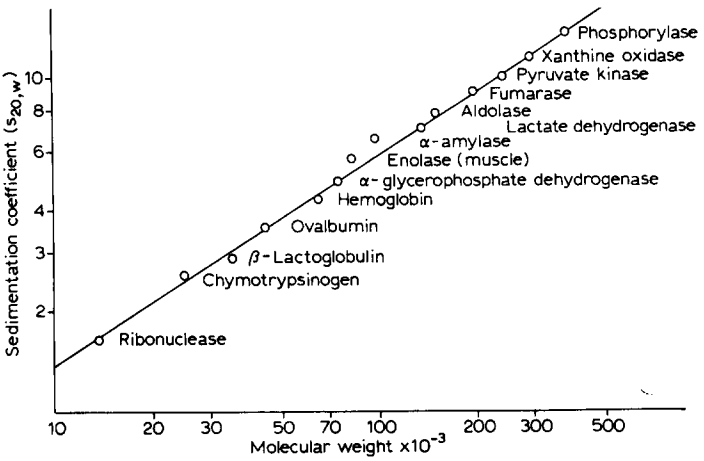

Fig. 4. Relationship between sedimentation coefficients of proteins and their molecular weights, using values obtained from the literature.

The results of the sedimentation velocity experiments were confirmed by sedimentation equilibrium experiments, using the Archibald[17] method modified for use in conjunction with absorption optics (see Methods). This method allows for the calculation of molecular weights in a very short time with a minimum of labor. At 25 °C and 10 μgm/ml, no dissociation occurred, with a value for the native dimer of 90 000–100 000 being obtained. This value corresponds to the value found by Mann *et al.*[8] and is considerably larger than the previously accepted value of 67 000 (Gawronski and Westhead[6]). Due to the limitations of the light source it was not possible to do experiments below 10 μg/ml. At 40 °C and 10 μg/ml the mol. wt is 53 000 corresponding to 80% monomer, thus verifying data obtained by sedimentation velocity.

Dissociation of yeast enolase also occurs at 40 °C in the presence of substrate, 2.44 mM 2-phosphoglycerate and 1 mM $MgCl_2$, but at a 200-fold lower concentration than in the absence of substrate. As shown in Fig. 5, 50% dissociation occurs at approx. 0.2 μg/ml. The 50% dissociation point is obtained by measuring the enzyme concentration at 4.8 S, the calculated sedimentation coefficient for a equal mixture of dimer and monomer. This is to be compared with the value of 40 μg/ml found in the absence of 2-phosphoglycerate. Both sets of experiments included 1 mM $MgCl_2$. Because of the very small amount of product formed at low enolase concentrations, it was not possible to do experiments below 0.07 μg/ml. These experiments were performed using the active enzyme centrifugation method (see Methods). A sedimentation coefficient of 3.6–3.8 S is obtained by extrapolation of the data in Fig. 5 to zero protein concentration. This value agrees very closely to the value of 3.7 found for the monomer at 40 °C (Fig. 3). Also the sedimentation values (5.75 S) obtained above 1 μg/ml correspond to the values obtained for native enolase using the formal means of measuring sedimentation coefficients. (Fig. 3). Since the amount of product formed is a function of the temperature of the reaction medium, it was not possible to do 25 °C centrifuge runs at the same low levels as at 40 °C. At the lowest level tested (0.27 μg/ml) enolase has a sedimentation coefficient of 5.73 S, indicating no dissociation has occurred. All sedimentation velocity experiments yielded a single

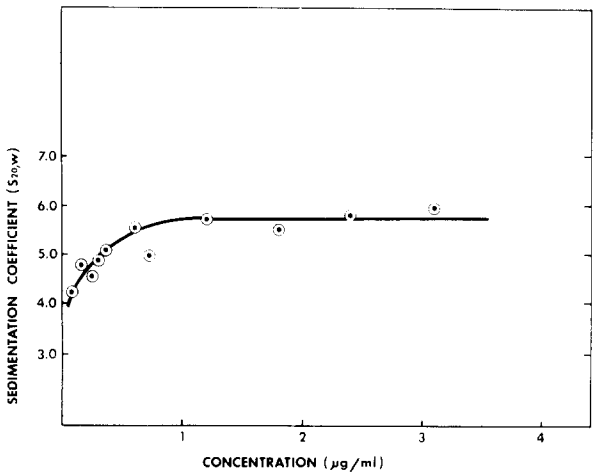

Fig. 5. Sedimentation coefficients of yeast enolase at 40 °C, obtained using the active enzyme centrifugation method, as a function of yeast enolase concentration. All centrifugations were carried out at 60 000 rev./min in 0.1 M Tris–HCl, 1.0 mM $MgCl_2$, 2.44 mM 2-phosphoglyceric acid and 5% sucrose. Sedimentation coefficients were measured by following the inflection point of the product distribution curve, phosphoenolpyruvate, at 240 nm.

symmetrical sedimenting front indicating a fast equilibrium between monomer and dimer.

The first conclusion one draws from the above data is that the monomer must be enzymatically active. However, it is possible that the dimer is the sole source of the enzymatic activity that is observed in the centrifuge cell, the monomer being completely inactive. If this is the case, the specific activity of the enolase should decrease as the amount of monomer increases, ultimately resulting in no activity when only monomer is present. However, if the monomer and dimer have the same

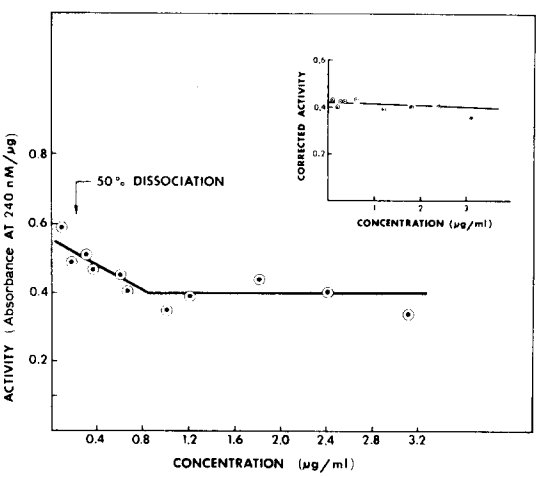

Fig. 6. The amount of phosphoenolpyruvate formed per μg of enolase (*i.e.* specific activity) as a function of enolase concentration. As the data used here are from the same centrifugation runs as shown in Fig. 5, the conditions are identical to those in Fig. 5. The inset represents the activities corrected for the difference in sedimentation rates between monomers and dimers (see text).

catalytic capabilities (on a μg basis), the specific activity should remain constant. Fig. 6, which plots the amount of phosphoenolpyruvate produced (measured as absorbance at 240 nm) per μg of enolase as a function of enzyme concentration, shows that the specific activity does not decrease as the amount of monomer increases. The increase in activity as the amount of monomer increases is due to the fact that as the sedimentation coefficient decreases, the time required for the band of enolase to pass a given point in the ultracentrifuge cell increases, resulting in the production of larger amounts of phosphoenolpyruvate. This increase in activity was corrected as shown below.

$$\text{Corrected activity}_c = \text{Activity}_c \cdot s_{20,w(c)}/s_{20,w(\text{native})} \quad (3)$$

where subscript c refers to the values obtained for activity and $s_{20,w}$ at concentration c and $s_{20,w(\text{native})}$ is equal to 5.85 S (Fig. 3). The inset in Fig. 6 illustrates this correction. As the activity remains constant as dissociation occurs one concludes that the dimer and monomer have equal catalytic capabilities.

DISCUSSION

By directly observing the enzyme–substrate complex it is possible to directly quantitate some of the physical parameters of the reactive species rather than measuring these parameters at concentrations many fold higher than physiological concentrations. Cohen *et al.*[20,21] used this method to determine sedimentation coefficients of enzyme–substrate complexes by determining the amount of product produced or substrate used up between successive scans. When they plotted the decrease or increase in absorbance as a function of cell radius, a gaussian-shaped curve was obtained for each successive pair of scans. These difference curves corresponded to the distribution of enzyme activity and thus defined the shape and position of the sedimenting band of enzyme. As used by Cohen *et al.*[20,21] these calculations are very laborious and can be eliminated if initial rate velocities are maintained at all times during the centrifuge experiment (*i.e.* the enzyme is saturated with substrate). This criteria is met by keeping the ratio of substrate to product high. Under such conditions the scanner trace of the product formed will correspond exactly to the shape and position of the sedimentating enzyme band. Sedimentation coefficients can then be determined by following the inflection point of the product distribution curve. Taylor *et al.*[22], in an extensive study of several pyruvate carboxylases obtained similar sedimentation coefficients using either the difference method of Cohen *et al.*[20,21] or the conventional inflection point method. The application of the difference method to yeast enolase also yielded sedimentation coefficients identical to those obtained by following the inflection point of the product distribution curve.

The condition of maintaining initial saturating substrate concentration is difficult to fulfill when one measures the disappearance of an absorbing substrate rather than the appearance of an absorbing product. However with the proper conditions it is possible to determine sedimentation coefficients when measuring the disappearance of substrate. Such an experiment was reported by Barlow *et al.*[23] using beef liver catalase as the enzyme and H_2O_2 as the ultraviolet-absorbing substrate. The kinetic difficulties were overcome by placing a 5-fold excess of substrate

in the sample side of the cell. As only a small amount of the total absorbance will disappear during the course of the centrifugation, making it very difficult to measure sedimentation, substrate is also placed in the reference side of the centrifuge cell. If the amount of substrate placed in the reference side of the cell approximates the amount of unreacted substrate, after completion of the sedimentation experiment, in the sample side of the cell, the scans obtained will be full scale when a small amount of substrate is reacted. Sedimentation coefficients obtained for catalase agreed with the values obtained by conventional means, thus proving that the kinetic consideration of maintaining initial rate velocity during the entire sedimentation experiment was met.

As the enolase monomers have complete and full activity one wonders as to what is the nature of the catalytic unit *in vivo*. The approximate concentration of enolase in the yeast cell is 0.1 mg/ml (Wold[24]) and as the point of 50% dissociation in the presence of the substrate 2-phosphoglyceric acid is 0.2 μg/ml, it is unlikely that an active monomer exists *in vivo*. Apparently the dimer must have some structural or catalytic advantage over the monomer. What this advantage is of course can not be answered from the experiments reported here but it does pose an interesting technical question. To the knowledge of this author there is only one other reported case of active monomers, that being phosphoenolpyruvate carboxytransphosphorylase (Haberland[25]). It may be that under proper dissociating conditions many multichain enzymes will be active in the monomer or dissociated state.

With the use of the Archibald technique in conjunction with the scanner optical system one can determine molecular weights in a matter of 2–3 h, including all calculations. This method has been extensively tested with proteins ranging in molecular weight from 13 000 to 260 000, and in all cases the molecular weights derived agreed within 1–4% of the literature values. The use of a computer program, which would accurately extrapolate the concentration curve to the meniscus, thus facilitating the determining of a very accurate value for dc/dr at the meniscus, should improve the accuracy of this method.

ACKNOWLEDGMENTS

The author would like to thank Dr S. Keresztes-Nagy for introducing me to this problem and for providing helpful discussion during the course of the experiments.

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