Glucose 6-Phosphate Dehydrogenase (Zwischenferment). II. Homogeneity Measurements and Physical Properties of the Crystalline Apoenzyme from Yeast*

Robert H. Yue, Ernst A. Noltmann,† and Stephen A. Kuby

ABSTRACT: The crystalline D-glucose 6-phosphate dehydrogenase, when freed of oxidized triphosphopyridine nucleotide, has withstood several criteria of purity. These criteria have included sedimentation velocity, sedimentation equilibrium, electrophoresis, and diffusion. A number of physical properties have been determined on the apoenzyme and a value for its molecular weight has been assigned, *viz.*, 102,000.

In the first paper of this series (Noltmann *et al.*, 1961) the isolation of crystalline D-glucose 6-phosphate dehydrogenase[1] from brewers' yeast was described. It is the purpose of this paper to report on some studies which bear specifically on the homogeneity of this protein preparation, when essentially freed of its coenzyme, TPN[2] (*i.e.*, the TPN-free species or the *apoenzyme*). Coincident with this work, a number of physicochemical properties of the isolated apoprotein have been determined. A preliminary report and a description of some early work have been given elsewhere (Yue *et al.*, 1966; Noltmann and Kuby, 1963).

A later communication will deal in some detail with the physical properties of the TPN–enzyme species and the phenomenon of the molecular association of the protein as revealed primarily by ultracentrifugation studies (Yue *et al.*, 1966; Noltmann and Kuby, 1963). Future studies will be concerned with the internal structure of the protein as deduced chemically and physically, and with the ultimate problem of correlating information, thus obtained, on the physical and chemical properties of the enzyme protein with the kinetics of its catalyzed reaction.

Materials and Methods

Preparation of the Crystalline Apoprotein. Glucose 6-phosphate dehydrogenase was isolated from brewers' yeast essentially by the procedure described by Noltmann *et al.* (1961), with minor modifications to permit isolation of the enzyme protein from initially 10 kg of dried brewers' yeast compared to the 3-kg scale previously employed. (Large quantities of dried brewers' yeast for enzyme work, the Anheuser-Busch strain of *Saccharomyces carlsbergensis*, were generously supplied by Anheuser-Busch, Inc., St. Louis, Mo. To facilitate the operations at this increased scale, some of the steps were simplified and a second use of DEAE-cellulose introduced; the details of these slight modifications have been described (Kuby and Noltmann, 1966) and only the procedure used for preparation of the apoenzyme will be mentioned.

After four to six crystallizations of the enzyme from aqueous $(NH_4)_2SO_4$ solutions containing TPN (Noltmann *et al.*, 1961), the enzyme preparations reached constant specific activity. Although several methods were tried initially to remove the TPN, without inactivation of the enzyme, the most successful method found involves use of dialysis at high ionic strengths, followed by crystallization of the apoenzyme, either from $(NH_4)_2SO_4$ solutions or crystallization at low ionic strength at a pH value near its apparent isoelectric point. Two to three preparations are combined and the crystals are collected by centrifugation. After dissolution of the crystalline pellet in 0.05 M phosphate (NH_4^+)–0.01 M EDTA (pH 6.1) to yield a 2–3% protein solution, it is dialyzed at 3° over a 2- to 3-day period against repeated changes, each of *ca.* 1000 volumes of a solution containing 0.05 M phosphate (NH_4^+)–0.01 M EDTA–2.145 M $(NH_4)_2SO_4$ (or 55% saturation) (pH 6.1). After centrifugation to remove any perceptible opalescence, the protein solution, which may still contain traces of TPN, is then induced to crystallize as the apoprotein at about 0.62 saturation $(NH_4)_2SO_4$.[3] (The solubility of the crystalline apoprotein in aqueous $(NH_4)_2SO_4$ solution is greater than the crystalline TPN–enzyme.) During the course of the next several days

* From the Laboratory for Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah, Salt Lake City, Utah. *Received December 7, 1966.* This work was supported in part by grants from the National Science Foundation and from the National Institutes of Health.

† Permanent address: Department of Biochemistry, University of California, Riverside, Calif.

[1] IUB systematic name: D-glucose 6-phosphate:NADP$^+$-oxidoreductase.

[2] Abbreviation used: TPN, oxidized triphosphopyridine nucleotide.

[3] $(NH_4)_2SO_4$ obtained either from Mann Research Laboratory or recrystallized analytical grade $(NH_4)_2SO_4$ (Mallinckrodt) is used.

the saturation $(NH_4)_2SO_4$ is increased to about 0.66–0.67, and 89–90% of the apoprotein will have crystallized. The crystals are collected by centrifugation, dissolved in 0.01 M EDTA–0.05 M phosphate (NH_4^+) (pH 7.4), and dialyzed *vs.* 0.05 M phosphate (NH_4^+)–0.01 M EDTA–2.145 M $(NH_4)_2SO_4$ (pH 6.8). Under these conditions, the enzyme solution is fairly stable, with no significant loss in activity over a 4-month period (at -5 to $-10°$). Aliquots of the apoprotein solution were then dialyzed exhaustively against the buffers employed in each of the measurements (a description of buffers may be found in the legends to the figures).

During the course of studying the electrophoretic properties, another method was discovered for crystallization of the apoprotein, which makes use of a gradual adjustment of ionic strength through dialysis, at a pH near its apparent isoelectric point. Aliquots of the protein solution (2–3%) in 55% saturation $(NH_4)_2SO_4$ are dialyzed *vs.* an 0.01 M imidazole (Sigma)–HCl buffer (pH 6.2); over the course of 1–2 days, the apoenzyme will crystallize in the dialysis bag as relatively enormous, but fragile, crystalline plates, which readily dissolve in 0.1 ionic strength buffers above pH 6.5. Interestingly, the crystalline shapes of the apoprotein (microscopic needles from $(NH_4)_2SO_4$ solutions, or plates from low ionic strength isoelectric solutions) are dramatically different from that of the bipyrimidal structure of the TPN–crystalline enzyme in $(NH_4)_2SO_4$ solutions (Noltmann *et al.*, 1961). There does not appear to be any increase in specific activity for the crystalline apoprotein preparations, but they usually appear to be free of TPN as judged from the ultraviolet absorption spectrum. It is convenient to monitor the ratio of absorbancy values at 278:259 mμ; and a ratio of 1.98 has usually been obtained for the "TPN-free" species. In one case, however, this ratio appeared to be slightly lower; and the protein preparation in 0.01 M EDTA (pH 6.8) was, therefore, repeatedly precipitated with $(NH_4)_2SO_4$ at 0.90 saturation and then redialyzed against 55% saturation $(NH_4)_2SO_4$–0.01 M EDTA (pH 6.8) as described above.

Other Materials. Chromatographed bovine pancreatic ribonuclease A, employed for many initial test studies, was obtained from Sigma Chemical Co. (lot 63B-8560-7, 126 Kunitz units/mg). Reagent grade Tris and TPN were of Sigma origin. Potassium dichromate, used in the calibration of the optical cuvets, was obtained from the National Bureau of Standards. All other reagents were the best available analytical grade commercial products. Twice-distilled deionized water was used in the preparation of all solutions for the physical measurements. (When necessary, any dissolved traces of CO_2 in the water were removed by boiling.)

Diffusion. Free diffusion coefficients (D) were determined with the use of a Spinco Model H electrophoresis diffusion instrument at 3.00°, with the light source filtered to yield 546 mμ. Both the new Spinco "one-piece" diffusion cell (*i.e.*, without movable sections, Spinco Division, Manual, 1965) and the modified 2-ml microelectrophoresis cell were employed; in the case of the latter cell, the two extension tubes were connected by side arms (Schachman, 1957), and the experimental procedures and method of boundary sharpening followed that described by Schachman (1957). Since there are apparently no reported uses of the new diffusion cell, as of the present time, it is perhaps of interest to compare it with the conventional modified Tiselius cell. The main advantages of the one-piece cell are the absence of sliding surfaces (and consequently the elimination of the use of lubricants) and the fact that it permits the use of a reference solution (for Rayleigh optics) other than that of the bath liquid; its optical path (four times that of the micro-Tiselius cell) is equivalent to the conventional 11-ml cell, but requires less than one-half the volume of solution. However, the boundaries tend to be less stable in the one-piece cell as compared to the micro-Tiselius cell, since the initially sharpened boundary, which is under observation, cannot be isolated. More care must be exercised in the use of the one-piece cell to ensure that the flexible tubing, which is connected to the lower filling plug, is secured and fixed in place, otherwise it will impart a certain degree of instability to the boundary; in addition, it has been found helpful to construct a loose-fitting cover for the extension tube to prevent contamination of the channels by droplets of condensed liquid, dust, etc. With careful use, the one-piece cell yields good results and interferograms of excellent quality (see Figure 1).

Diffusion coefficients were calculated, for the most part, from photographs (on metallographic plates) of the Rayleigh interferograms. Computations were made by the method of Longsworth (1952); the tables of probability functions employed in the calculation were originally obtained from the Federal Works Agency, Superintendent of Documents, Washington, D. C. (1941). The measured values of diffusion coefficients so obtained ($D'_{measured}$) were then corrected for the uncertainty in the "zero time" by means of the expression $D'_{measured} = D(1 + (\Delta t/t'_{measured}))$, where D is then $D_{3°,b}$, for 3° and buffer (Longsworth, 1947). Diffusion measurements were initially made on both sucrose and bovine albumin (Armour, lot no. V-68802) solutions as a check on the procedure. For an 0.500% (w/v), sucrose solution in water (prepared according to Gosting and Morris, 1949), a value of $D_{3°,w} = 2.612 \times 10^{-6}$ cm²/sec was obtained, which may be compared with a value of $D_{3°,w} = 2.605 \times 10^{-6}$ cm²/sec calculated from the data at 1° ($D_{1°,w} = 2.418 \times 10^{-6}$ cm²/sec) of Gosting and Morris (1949). Similarly, good agreement with the data of Creeth (1958) on bovine plasma albumin was obtained.

To convert $D_{3°,b}$ "to standard conditions" the Stokes–Einstein relation was employed together with the use of measured values of viscosity, to yield $D_{20,w}$. Apparent weight-average molecular weights, from diffusion, sedimentation velocity (see below), and $\bar{V}_{a,p}$ data were then obtained from the Svedberg relation, $M_{s,D} = RTS/D(1 - \bar{V}\rho)$. Since Gosting and Morris (1949) have found very slight inherent errors in the Stokes–Einstein relation, calculations

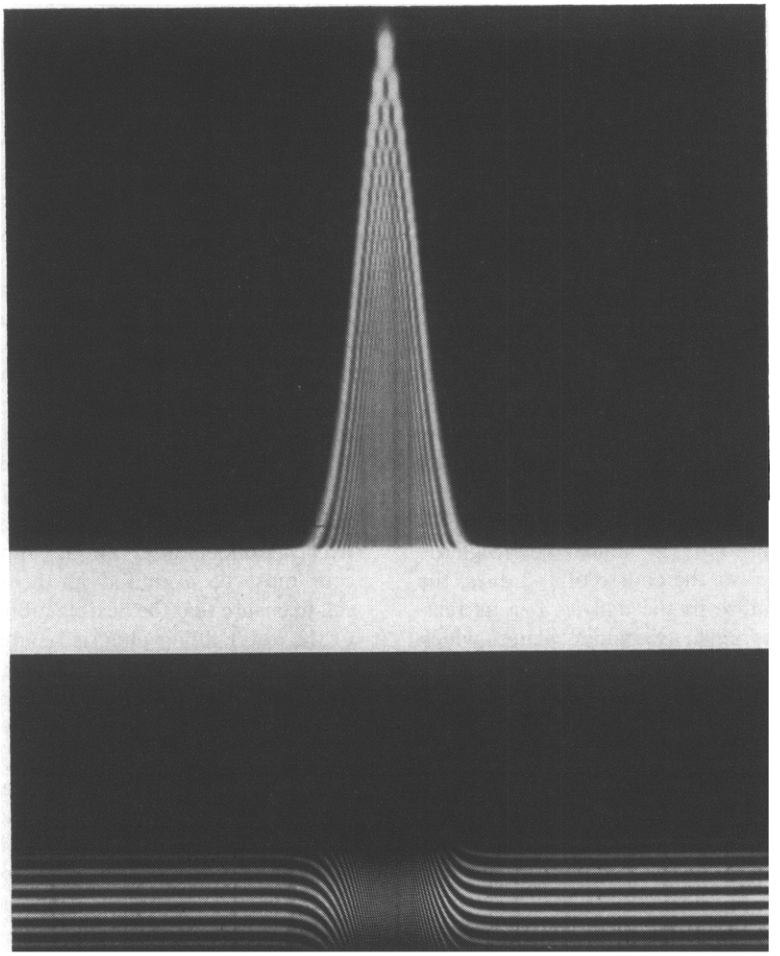

FIGURE 1: A free diffusion pattern at 3.00° of TPN-free glucose 6-phosphate dehydrogenase obtained with the Spinco one-piece diffusion cell. The initial protein concentration was 2.48 mg/ml (J_0 = 41.27 Rayleigh fringes) in 0.15 M NaCl–0.01 M phosphate–0.01 M EDTA (pH 6.8) at 3.0°. Time = 16,395 sec after boundary sharpening. The schlieren pattern is shown at the top (schlieren diaphragm angle set at 70° with a knife edge as diaphragm). The Rayleigh interference pattern is given at the bottom.

of $M_{s,D}$ were made as well from the $s_{3°,b}$, $D_{3°,b}$, and $\bar{V}_{app,3°}$ sets of data.

Sedimentation velocities were measured at 3° in a Spinco Model E analytical ultracentrifuge, equipped with an RTIC temperature-control system and a phase plate as a schlieren diaphragm (the schlieren optical system was aligned according to Gropper, 1964). Kodak metallographic plates were employed to photograph the schlieren patterns with a Kodak Wratten 77A filter over the light source. For most of the work a single-sector cell with 12-mm optical path was employed. Sedimentation coefficients at 3° and buffer ($s_{3°,b}$) were calculated in the usual fashion from log x *vs.* t plots (Schachman, 1957), and converted to $s_{20,w}$ values with the use of measured values for the densities and viscosities of the buffers employed and values for the partial specific volume of the protein (Svedberg and Pedersen, 1940). For the purpose of extrapolating the sedimentation coefficients to zero-protein concentration, a linear plot was made against the average boundary concentrations during the run.

Sedimentation equilibrium experiments were performed also at 3° with the use of a rotatable light source and Rayleigh interferometric optics (Richards and Schachman, 1959); the optics were also aligned according to Gropper (1964). A slow stream of air was passed over the outside of the lower collimating lens to eliminate moisture condensation during periods of centrifugation (Yphantis, 1964). A double-sector, synthetic boundary cell, of the capillary type, with sapphire windows and 12-mm optical path was employed. Kodak IIG plates were used for recording the interferograms.

During the initial phases of the work, a dense liquid, FC43 (Yphantis, 1964), was used to provide a transparent cell bottom of the proper shape (Ginsberg *et al.*, 1956); but in later runs, and for the work reported here, it was omitted when evidence of aggregation was obtained in a few cases and other difficulties encountered with its use.

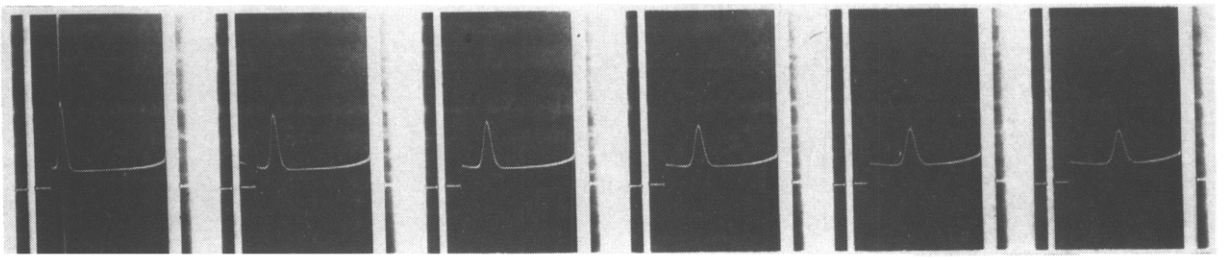

FIGURE 2: Sedimentation velocity patterns of TPN-free glucose 6-phosphate dehydrogenase. Exposures represent a single determination on six-times-crystallized enzyme (five times as the TPN–enzyme and once as the apoprotein); see Experimental Section) in 0.15 M NaCl–0.01 M sodium phosphate–0.01 M EDTA (pH 6.8) at 3°. Sedimentation (in a single-sector, 12-mm cell) proceeds to the right at 16-min intervals, 59,780 rpm, and an average temperature of 3.3°; the average protein concentration at the boundary is 7.12 mg/ml. The schlieren patterns were obtained with the schlieren diaphragm (phase-plate) angle set at 75°.

The experimental procedure followed that described (Spinco Division, 1964), with slight modifications. A calibrated micropipet fitted with polyethylene capillary tip was used to fill the solution sector of the cell (137 μl),[4] and the total number of Rayleigh diagonal fringes of the solution (*i.e.*, the initial concentration, J_0, in fringes) was determined first by layering the dialysate liquid over the enzyme solution at the same speed to be used in the final equilibrium run, in order to minimize the error due to cell distortion. The cell was then emptied of its contents and carefully cleaned, without dismantling, in order to retain the same optical path for the sedimentation equilibrium experiments; the cavities were then refilled with protein solution (137 μl) and buffer (*ca.* 150 μl) with the use of micropipets, to yield a 4-mm liquid column for the solution side. To reduce the time required to reach sedimentation equilibrium, the rotor (An-D or AN-E) was overspeeded for a calculated period of time followed by reduction in speed to the desired equilibrium speed (Hexner *et al.*, 1961), which was selected to provide a three- to fourfold ratio in (c_b/c_m), concentration at the cell bottom to that at the meniscus (Svedberg and Pedersen, 1940). The legends to the figures give the pertinent information.

To translate the fringe patterns into an expression relating concentration to distance, the technique of continuous photography (based on that described, Spinco Division Manual, 1964) was employed to locate the position of the hinge point. With the one level in the cell corresponding to the initial concentration located, and coupled with the value of the initial concentration (J_0, in fringes) from the synthetic boundary run, a straight-forward calculation of the concentration of the protein (J_x, in fringes) at various radial distances (x) in the cell followed. Initially, the technique was checked by making use of calculations based on equations expressing the conservation of mass within the cell (Richards and Schachman, 1959). Sedimentation equilibrium was presumed to be reached only when all the fringes throughout the liquid column were stationary for at least 12 hr, within the limits of detection of the two-coordinate microcomparator (Gaertner) employed.

The apparent weight-average molecular weights at various positions in the cell at equilibrium, were calculated from ln J_x *vs.* x^2 plots, according to $M_{wx} = 2RT[\mathrm{d}(\ln c_x)/\mathrm{d}(x^2)]/[(1 - \bar{V}\rho)w^2$ (where c_x was expressed in J_x fringes, and the other symbols bear their usual meaning); and the mean z-average molecular weight for the entire cell contents was calculated from the limiting slopes of the ln J_x *vs.* x^2 plots at the cell bottom and meniscus and with the use of the equation $\bar{M}_z = (M_{wx_b}C_b - M_{wx_m}C_m)/(C_b - C_m)$ (Svedberg and Pedersen, 1940).

Solutions of 5 mg/ml of ribonuclease (Sigma lot 63B 8560-7) and 4-mm liquid columns were used as an over-all check on the procedure and yielded an apparent weight-average molecular weight of 13,800, which may be compared with a value of 13,683 calculated from its known sequence and amino acid composition (Stein *et al.*, 1956; Smyth *et al.*, 1963).

Refractive Index Increments. Coincident with these studies, both the Model H and Model E instruments were employed as Rayleigh interferometers to measure the refractive index increment of the protein at 546 mμ. The optical paths of the cells were measured with the use of sucrose solutions whose refractive index increments are known to a high degree of accuracy. A 0.750% sucrose solution (w/v) was prepared just prior to the experiment, in accordance with the directions of Gosting and Morris (1949). The optical paths of the cells for either instrument were determined with this sucrose solution, just before determinations were made on the protein solution, with the use of the refractive index increments estimated from the data of Gosting and Morris (1949) (*i.e.*, $(\Delta n/\Delta c)_{3°} = 1.4653 \times 10^{-3}$) and the following equation $l = J\lambda/c(\Delta n/\Delta c)$ (where l is the optical path in centimeters; λ, the wavelength in centimeters; c, the concentration in

[4] The volume of protein solution used in the layering experiment is close to that which LaBar (1966) has also found to yield minimal errors.

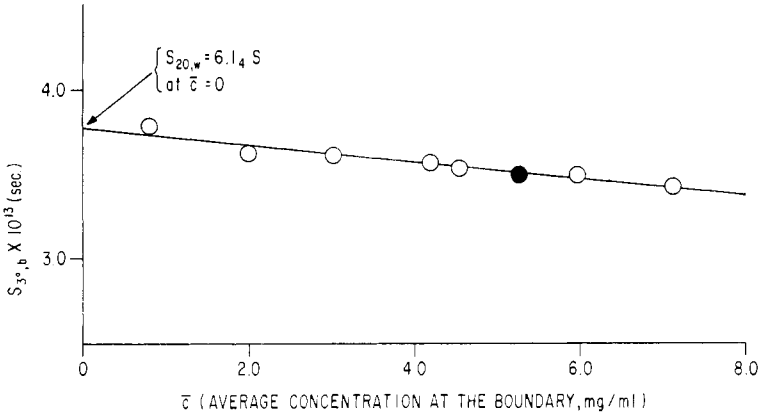

FIGURE 3: Sedimentation coefficients of glucose 6-phosphate dehydrogenase apoprotein as a function of protein concentration at pH 6.9 and 3°. Concentrations at the boundary (c) in milligrams per milliliter are the average values between the beginning and the end of the experiment, as determined by correcting for the radial dilution of the sector-shaped cell. (○) Protein dialyzed against 0.15 M NaCl–0.01 M sodium phosphate–0.01 M EDTA. (●) Protein dialyzed against 0.15 M NaCl–0.01 M EDTA. The linear least-square equation corresponding to the straight line drawn is $S'_{3°,b} = 3.77 \times 10^{-13}$ sec $(1 - 0.013c)$.

g/100 ml; J, the differential number of Rayleigh fringes; and $(\Delta n/\Delta c)$, the refractive index increment of the solution in (g/100 ml)$^{-1}$). In the case of the ultracentrifuge cell (a double-sector, 12-mm, synthetic boundary cell), it was cleaned without dismantling; and for both the modified Tiselius cell (2 ml) as well as the centrifuge cell, the protein solutions were filled to precisely the same heights as those used for the sucrose solutions, in the determination of the optical paths (l). The values for $(\Delta n/\Delta c)$ at 3° (and 546 mμ) when measured in this fashion in both instruments, with the same protein solution, were found to agree within 0.2%.

Electrophoresis. Electrophoretic (moving boundary) measurements were conducted in the Spinco Model H instrument at 1° with the 2-ml micro-Tiselius cell and schlieren optics. Solutions of protein were exhaustively equilibrated by dialysis for 24–36 hr against buffers at various pH values and ionic strengths (see legend to Figure 6). Conductivity measurements at 0.0° were made on the equilibrated buffer, with the use of a Radiometer conductivity meter, type DCM-2d, and a refrigerated bath (Precision Scientific Instruments, Temptrol bath). The calculated electrophoretic mobilities are consequently those for 0° (Tiselius, 1937). Migration of the boundaries was measured from the center line of the boundary with a Gaertner micro-comparator and calculation of mobilities were made in the conventional manner (Abramson *et al.*, 1942; Longsworth, 1942) on the descending boundary. To minimize deviations from ideality and convective disturbances in the cell, relatively dilute protein concentrations (which ranged from about 1 to 5 mg/ml) and comparatively low electric field strengths (which were never higher than about 6 v/cm) were employed for 0.1 ionic strength buffers (and the 2-ml cell). "False boundaries" were not too pronounced on well-dialyzed samples. For most cases, the patterns appeared to be enantiographic and symmetrical; asymmetry of the descending boundary (but not in the ascending boundary) was noticeable in the one case of 0.05 (Γ/2) phosphate buffer, at a pH about 7.9 and at a relatively high protein concentration, a phenomenon which is likely due to interaction with the phosphate buffer species (see Results and Discussion). The pH values of the buffers were measured at 3° with a Radiometer pHM 22p meter, equipped with scale expander, type pHA, and A. H. Thomas glass-calomel electrodes.

Partial Specific Volumes. Apparent partial specific volumes were determined pycnometrically. The pycnometers were of the self-adjusting capillary type blown by the Microchemical Specialties Co. according to the design of Anderson (1948), with slight modifications to include two glass sleeves with ground microjoints which fitted to each of the capillary tips of the pycnometer. These sleeves provided the means of filling the pycnometer (by gentle suction) and collection of any expelled microdroplets during the weighing period at the temperature of the balance. The pycnometers were calibrated with water, and the volume for the 1-ml pycnometer employed, for example, was found to be 0.96481 ml at 3° and 0.96505 ($\pm 8 \times 10^{-6}$) ml at 20.0°. Baths at 3.0 and 20.0 ± 0.05° were used to temperature equilibrate the filled pynocmeters, and weighings of the filled and temperature-equilibrated pycnometers were made against an empty pycnometer (for a counterpoise), with an Ainsworth Magni-Grad, type FHM, microbalance (sensitivity of 0.001 mg). The final weights were obtained by extrapolation to zero time to correct for any evaporation of the microdroplets in the glass sleeves, and the final weights were then corrected to *in vacuo*. Density measurements were carried out on protein samples equilibrated, by dialysis, against the buffers employed for the physical

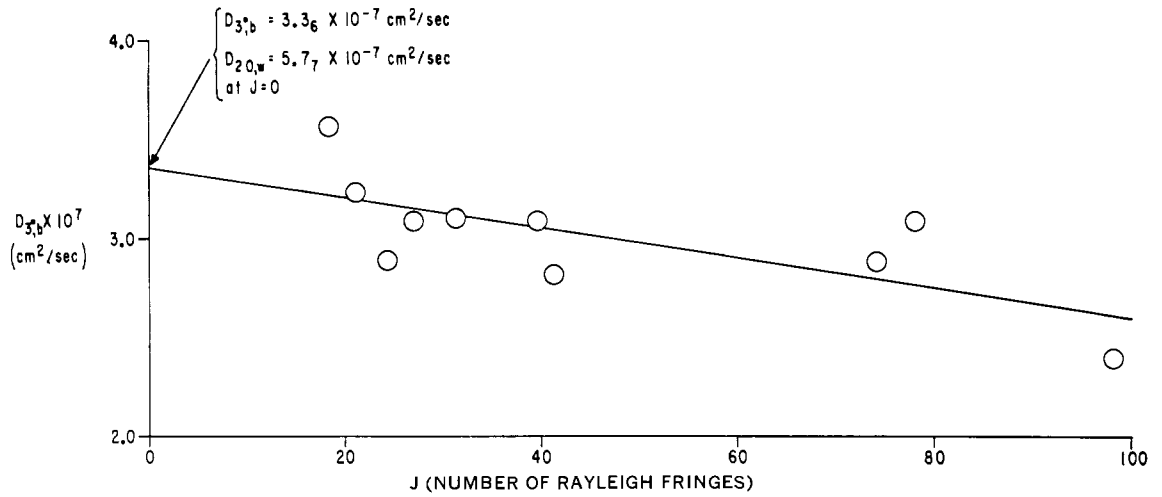

FIGURE 4: Diffusion coefficients of glucose 6-phosphate dehydrogenase apoprotein as a function of protein concentration, at 3.00° (pH 6.9), 0.15 M NaCl–0.01 M sodium phosphate–0.01 M EDTA. Data were obtained in both the one-piece cell and modified micro-Tiselius cell. The concentrations have been expressed in terms of Rayleigh fringes (J) across the protein boundary, corrected for differences in optical paths of the two cells, and referred to that of the one-piece cell. The initial protein concentrations thus ranged from about 1.2 to about 6.3 mg/ml over the entire plot.

measurements and on the buffers themselves. From these values and the weight fractions of the proteins (see below), the apparent partial specific volumes were calculated according to Kraemer (1940).

Other Methods. Weight fractions of the protein solutions, which were required for the refractive index increment and partial specific volume measurements, were determined by drying aliquots (at approximately 74°) in tared microplatinum boats, to constant weight in an evacuated (0.05 mm) pistol tube, containing P_2O_5. Weighings were carried out to the nearest 0.001 mg. Ash was determined on these dried samples in the platinum boats by igniting in a double-porcelain crucible over a Meker burner. Aliquots of the dialysis fluids were subjected to the identical treatment (and at the same time) and the final dry weights (and ash) of the protein samples were determined by difference.

Measurements of the ultraviolet absorption spectra and biuret values (Gornall *et al.*, 1949) were made on aliquots of the same protein solutions whose weight fractions were determined. This permitted a calculation of extinction coefficients and biuret factors, which were then employed as secondary measures of the dry weight of the protein.

Spectral measurements were made with either a Beckman DU or Cary 14 spectrophotometer. The optical paths of the quartz spectrophotometer cuvets were calibrated with an alkaline solution of potassium chromate as described by Haupt (1952). Values for the extinction coefficients reported here were made in 0.05 M sodium phosphate (pH 7.00) at 25°.

Results and Discussion

Sedimentation Velocity. The glucose 6-phosphate dehydrogenase apoprotein appears to sediment as a single component with no evidence of heterogeneity (see Figure 2 for a typical sedimentation velocity run). The observed sedimentation coefficients (at 3° and buffer) appear to decrease very slightly with an increase in protein concentration (Figure 3), according to the expression $s_{3°,b} = 3.77 \times 10^{-13}(1 - 0.013\bar{c})$, where $\bar{c}$ is expressed in milligrams per milliliter of the average boundary concentration. This small concentration dependency over the concentration range explored, might be interpreted as indicative of the absence of an association–dissociation equilibria under these conditions (Schachman, 1959) and a negative slope has been considered to be characteristic of compact globular macromolecules (Schachman, 1959). An estimation of its frictional ratio (*vide infra*) might be considered as lending further support to this idea. However, the range of protein concentrations investigated in these sedimentation velocity studies may have been too narrow to reveal the effects of association–dissociation equilibria, and a slight negative slope in the $s_{3°,b}$ *vs.* $\bar{c}$ plot, over this limited concentration range, might not preclude weak interactions (Nichol *et al.*, 1964).

Extrapolation to zero-protein concentration yields a value of $s^0_{3°,b} = 3.77 \times 10^{-13}$ sec or an $s^0_{20°,w}$ (*i.e.*, after correction to the reference states of 20° and water) equal to 6.12 S.

Free Diffusion and Molecular Weight by Sedimentation Velocity Diffusion ($M_{s,D}$). The free diffusion coefficients were measured under identical conditions (of temperature, buffer species, pH, and ionic strength) as that employed for the sedimentation velocity studies. Under these conditions of measurement, the protein appeared to diffuse as a single component with no evidence of other protein components with widely differing diffusion coefficients. The diffusion data are

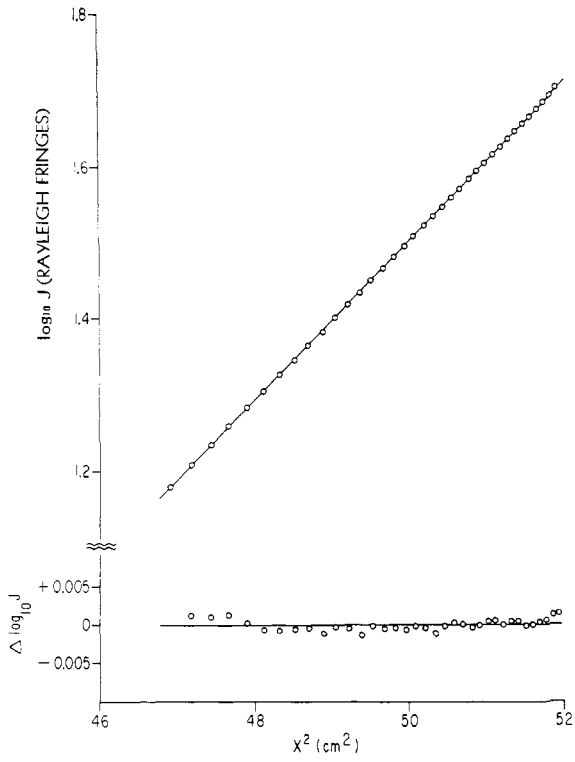

FIGURE 5: A molecular weight determination of glucose 6-phosphate dehydrogenase apoprotein from a sedimentation equilibrium experiment, expressed in terms of a plot of log J (log of the protein concentration in Rayleigh fringes) *vs.* x^2 (square of the distance, in centimeters squared, from the axis of rotation). The initial concentration of protein was 7.05 mg/ml, in 0.15 M NaCl–0.01 M sodium phosphate–0.01 M EDTA (pH 6.9) at 3.0°. The protein was sedimented initially at 11,250 rpm for 3.5 hr and then the speed was reduced to 6166 rpm. The time for the data given here was 92.5 hr after the equilibrium speed (6166 rpm) was reached at 3.0°, and no significant differences were noticed between 69 and 92.5 hr. A calculated value of M_{equil} (or $\bar{M}_w$) = 103,100 is obtained from these data, and $\bar{M}_z/\bar{M}_w = 1.013$. For the lower "deviation" plot: $\Delta(\log J)$ is the difference, at given values of x^2, between the least-square straight line and the experimental points.

summarized in Figure 4, in a plot of the diffusion coefficients *vs.* the total number of Rayleigh fringes (which in turn are a measure of the concentration across the protein boundary). The data fit the linear statistical relationship; $D_{3°,b} = 3.36 \times 10^{-7} - 7.67 \times 10^{-10}J$, with a typical dependency of D on concentration. An extrapolated value to zero protein concentration of $D^0_{3°,b} = 3.36 \times 10^{-7}$ cm²/sec is obtained, which, after correction to 20° and water gives a value of $D^0_{20°,w} = 5.77 \times 10^{-7}$ cm²/sec. (Under these conditions, the refractive index increment at 546 mμ, 3°, was determined to be 1.82×10^{-3} (g/100 ml)$^{-1}$.) Application then of the classical Svedberg equation for sedimentation velocity and diffusion together with measured values of the apparent partial specific volume of the protein (see below), gives a calculated value of the apparent weight-average molecular weight of 101,600 (with an estimated uncertainty of about 4900).

Sedimentation Equilibrium and Molecular Weight. Confirmation of the above value for the molecular weight was made by true sedimentation equilibrium analyses, and a typical analysis is presented in Figure 5, in a plot of log J *vs.* x^2, at sedimentation equilibrium. The linearity of the plot attests to the homogeneity of the preparation; the internal precision of the data, as illustrated by the lower deviation graph (Richards and Schachman, 1959) in Figure 5 is satisfactory and only near the very extremes of the liquid column do significant errors in measurement become manifest. The ratio of the z-average molecular weight to the weight-average molecular weight, for all the runs made, are close to the theoretical value of 1.0 (1.013 for Figure 5) for the case of a single macromolecular species.

In Table I, several data are presented for the apparent weight-average molecular weights ($\bar{M}_w$) as measured by sedimentation equilibrium for several initial protein concentrations. Over the relatively narrow initial protein concentration range which could be conveniently explored by this technique, there is no significant trend, and a mean value of 101,700 ± 2300 (*i.e.*, ±2 standard deviations) is obtained which is in excellent agreement with the value of $M_{s,D}$ obtained. However, since the range in concentrations explored would not be sufficient for an examination of any possible nonidealities of the solutions (Schachman, 1959), it is conceivable that this value for M_{equil} may be less accurate than the precision of the data would indicate.

TABLE I: Molecular Weight Determinations of Glucose 6-Phosphate Dehydrogenase Apoprotein by Sedimentation Equilibrium at 3.0° and pH 6.9, 0.15 M NaCl–0.01 M Sodium Phosphate–0.01 M EDTA, at Several Initial Protein Concentrations (expressed in Rayleigh fringes).

Initial Concn of Protein (Rayleigh fringes)	M_{equil} (or $\bar{M}_w$) (g/mole)
28.18	103,100
23.38	101,600
17.15	100,300
10.00	101,700
	Mean 101,700 ± 2300 (2 std dev)

Liquid Boundary Electrophoresis Studies. Examination of the electrophoretic behavior of the protein has revealed that, provided ion–protein binding is

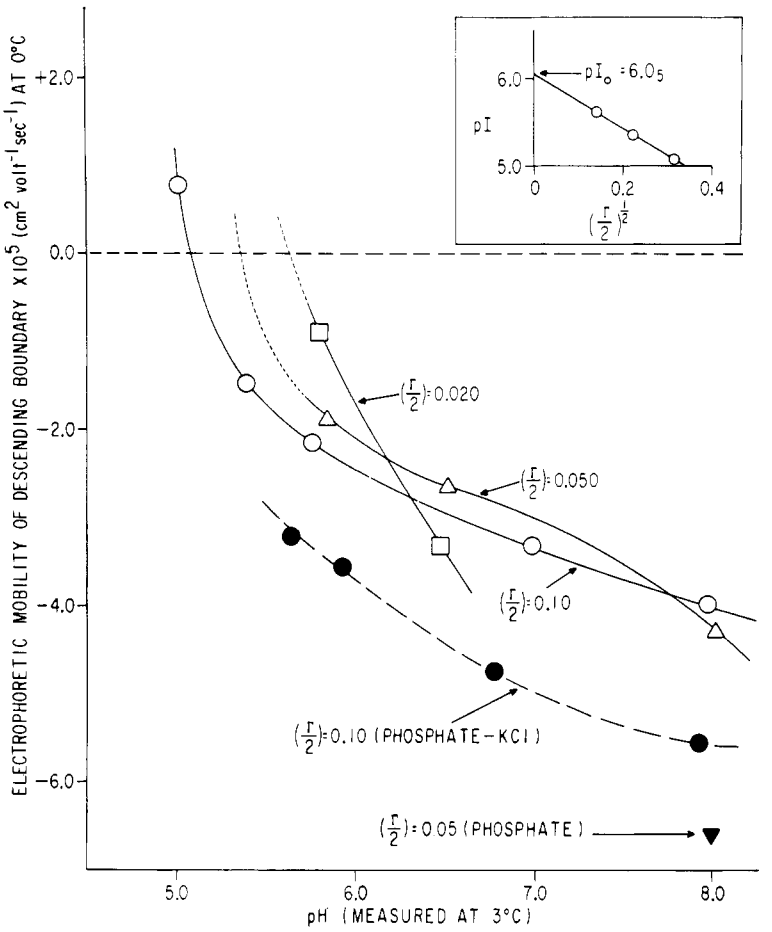

FIGURE 6: Electrophoretic mobility for 0° of glucose 6-phosphate dehydrogenase apoprotein as a function of pH (measured at 3°) at several ionic strengths. Lower graph: (○—○) 0.1 ionic strength buffers containing 0.05 ionic strength KCl (except for pH 5.71 buffer where 0.0725 ionic strength KCl was employed); buffer: Tris-KCl-HCl, imidazole-KCl-HCl, and potassium acetate–KCl–acetic acid at pH 7.98, 6.99, and 5.02, 5.71, and 5.94, respectively. (△—△) 0.050 ionic strength buffers; buffer: Tris-HCl, imidazole-HCl, and potassium acetate–acetic acid at pH 8.01, 6.51, and 5.83, respectively. (□—□) 0.020 ionic strength buffers; buffer: imidazole-HCl and potassium acetate–acetic acid at pH 7.51 and 5.80. (●—●) 0.10 ionic strength phosphate-KCl buffers containing 0.05 ionic strength KCl and 0.05 ionic strength phosphate: pH 7.93, 6.79, 5.92, and 5.62. (▼) 0.05 ionic strength phosphate (without KCl) buffer, pH 7.99. Upper graph: p*I*, isoelectric point, *vs.* $(\Gamma/2)^{1/2}$, square root of ionic strength.

slight, the enzyme migrates in an electrical field as a single component, over the range of pH values and ionic strengths explored. A summary of the data obtained is presented in graphical form in Figure 6, as a series of plots of mobility *vs.* pH. Data at the low ionic strengths could not be extended beyond the range given, because of the tendency of the protein to crystallize near its apparent isoelectric point, a condition which then led to dialyzed solutions which were too dilute for electrophoretic studies. At pH values more acid than the apparent isoelectric points, the enzyme proved to be unstable. Therefore, there is some uncertainty at the low ionic strengths in the extrapolation of the mobility data to a pH value of zero mobility. Nevertheless, it is evident that the isoelectric state (defined as the point of zero mobility) is a function of the ionic strength. On the upper portion of Figure 6, the isoelectric points are plotted as a linear function of the $(\Gamma/2)^{1/2}$, and by extrapolation to zero ionic strength a value of $pI_0 = 6.05$ is obtained. Studies on the amino acid composition of the protein (which are in progress) may reveal, whether this hypothetical value for pI_0, is close to a theoretical isoionic point.

In addition, the observed electrophoretic mobilities are also a function of the buffer species to some extent, since the mobilities as measured in buffers containing phosphate-KCl or phosphate alone ($\Gamma/2$ 0.1 or 0.05, respectively, but 0.05 $\Gamma/2$ in phosphate under all conditions) are far more negative than those measured in Tris, imidazole, or acetate buffers (with or without KCl) at the same pH values and ionic strengths (see Figure 6). Thus, there would appear to be a strong interaction between the protein and the phosphate

buffer species, in the region of the pH explored; in one case, at pH 7.99 and 5 mg/ml of protein, asymmetry of the descending schlieren pattern was observable, but not in the ascending limb. At lower pH values, or at lower protein concentrations, the patterns remained enantiographic and symmetrical. It is perhaps not surprising that phosphate strongly interacts with the apoprotein in view of the long-known inhibitory effects of phosphate on the catalyzed reaction (Theorell, 1935; Glazer and Brown, 1955). Recently, Barlow and Margoliash (1966) have reported quite dramatic effects of buffer species interactions with the cytochromes *c* as revealed by liquid boundary electrophoretic studies; and Cann and Goad (1964, 1965), Longsworth (1959), and others have discussed in detail both theoretically and experimentally the phenomenon of interactions between buffer species and protein during electrophoresis. Whether electrophoresis may be successfully employed as a quantitative tool to evaluate this complex type of ion–protein interaction, must await further information, at least on its titration behavior and amino acid composition.

TABLE II: Physical Properties of TPN-Free Glucose 6-Phosphate Dehydrogenase.

pI_0, isoelectric point ($(\Gamma/2) \rightarrow 0$)	6.05
$s^0_{3°,b}$, sedimentation coefficient ($\bar{c} \rightarrow 0$, pH 6.9) (sec)	3.77×10^{-13}
$s^0_{20°,w}$, sedimentation coefficient ($\bar{c} \rightarrow 0$, pH 6.9) (sec)	6.14×10^{-13}
$D^0_{3°,b}$, diffusion coefficient ($c_0 \rightarrow 0$, pH 6.9) (cm^2/sec)	3.36×10^{-7}
$D^0_{20°,w}$, diffusion coefficient ($c_0 \rightarrow 0$, pH 6.9) (cm^2/sec)	5.77×10^{-7}
$M_{s,D}$, molecular weight by sedimentation and diffusion ($c \rightarrow 0$) (g/mole)	101,600 ± 4900
M_{equil}, molecular weight by sedimentation equilibrium (g/mole)	101,700 ± 2300
$\bar{V}_{app,3°}$, apparent partial specific volume (c = 1.90%) (cm^3/g)	0.739
$\bar{V}_{app,20°}$, apparent partial specific volume (c = 1.90%) (cm^3/g)	0.744
f/f_0, molar frictional ratio	1.17 (a:b = 3.6)
$(\Delta n/\Delta c_{3°})$, refractive index increment (λ 546 mμ) (g/100 ml)$^{-1}$	1.82×10^{-3}
Biuret factor (λ 540 mμ, 10-ml volume, 1-cm light path) (mg/absorbance unit)	38.2
$E^{1\%}_{1\,cm}$, extinction coefficient (278 mμ, pH 7.00, 0.05 M sodium phosphate)	9.65
Ratio of extinction coefficients at 280:260 mμ	1.92
Ratio of extinction coefficients at 278:259 mμ	1.98

A summary of all the physical properties pertinent to this report is given in Table II. These data also include a measured value at 278 mμ for its ultraviolet extinction coefficient, at its absorption maximum; its biuret factor; its apparent partial specific volume, at 3 and 20°; and a calculated value for its molar frictional ratio. With the use of Perrin's (1936) equation for a prolate ellipsoid of revolution, it may be calculated that an f/f_0 value of 1.17 would correspond to an axial ratio of 3.6 for the major:minor axes (a:b) of an assumed anhydrous prolate ellipsoid of revolution. If the protein binds a typical amount of hydrated water, it would appear likely that this protein fits the category of a compact and globular macromolecule. A further discussion on this point will be postponed until a forthcoming report, when data will be presented on the effect of TPN, the presence of which profoundly affects the hydrodynamic properties of the protein (Yue *et al.*, 1966; Noltmann and Kuby, 1963).

In summary, the preparation of glucose 6-phosphate dehydrogenase apoprotein has withstood the several criteria of purity to which it has been subjected: (a) sedimentation velocity, (b) sedimentation equilibrium, (c) agreement in molecular weights by sedimentation velocity diffusion and sedimentation equilibrium, and (d) electrophoresis in weakly interacting buffers. A molecular weight for the apoprotein of approximately 102,000 has been obtained by the two procedures employed.

In view of the subunit or polymeric structure shown for several dehydrogenases, including the dimeric liver alcohol dehydrogenase, the tetrameric yeast alcohol dehydrogenase, and the tetrameric muscle triosephosphate dehydrogenase (refer to a review by Harris (1964) for pertinent references), an effort is currently underway to explore the possibility of an associated or subunit structure in the Zwischenferment molecule.

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Evidence for an Intermediate in the Acetylation Reaction of Acetylcholinesterase*

R. M. Krupka

ABSTRACT: Acetylation of acetylcholinesterase by the substrate methyl acetate was found to be controlled by an ionizing group in the enzyme of pK = 5.3. The reverse reaction (acetyl enzyme + methanol → methyl acetate), which was studied by hydrolyzing acetylcholine in the presence of 5% methanol and determining the ratio of hydrolysis to methanolysis, was controlled by another ionizing group, pK = 6.3. It follows that an intermediate, whose formation and decomposition are catalyzed by different enzyme groups, must occur in acetylation. In the direction of ester hydrolysis, the rate-limiting step is catalyzed by a group of pK = 5.3, and a subsequent fast step is catalyzed by a group of pK = 6.3. The latter group, which is adjacent to the enzyme's anionic site, also functions in the rate-limiting step of deacetylation.

Recently published evidence showed that different catalytic basic groups in AChE[1] function in the rate-limiting steps of each of the two main stages of substrate hydrolysis, acetylation of the enzyme, and deacetylation (Krupka, 1966a,b). The study of acetylation has now been carried further, with investigation of the pH dependence of the forward reaction, where an ester reacts with the enzyme to produce alcohol and an acetyl enzyme, compared with the back reaction, where the acetyl enzyme and alcohol react to form an

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[1] Abbreviations used: AChE, acetylcholinesterase; AcCh, acetylcholine bromide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.