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Effect of D_2O and Nicotinamide Adenine Dinucleotide on the Sedimentation Properties and Structure of Glyceraldehyde Phosphate Dehydrogenase†

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ABSTRACT: The binding of successive nicotinamide adenine dinucleotide (NAD) molecules to the subunits of rabbit muscle glyceraldehyde phosphate dehydrogenase was found by the technique of difference sedimentation to cause an inherent change in the sedimentation coefficient of the protein beyond that attributable to the buoyant weight of the coenzyme. Attempts were made to determine whether the measured changes in sedimentation coefficient were wholly or partially due to shifts in the association–dissociation equilibria between the enzyme and its subunits. The results were equivocal because aqueous preparations of the apoenzyme, produced by charcoal treatment, showed with time increasing amounts of subunits and aggregates larger than tetramers. This spontaneous conversion was accompanied by the loss of potential enzymic activity. Marked stabilization of the apoenzyme was achieved through the use of buffered D_2O solutions. The spontaneous inactivation and precipitation observed in aqueous solutions were strikingly eliminated in D_2O solutions even at room temperature. In addition, for most preparations of the enzyme in the absence or presence of NAD only tetramers could be detected by sedimentation equilibrium measurements over the concentration range from 0.1 to 3.0 g per l. This finding was confirmed by differential sedimentation studies which provided direct measurements of the concentration dependence of the sedimentation coefficient. For those preparations showing no evident dissociation in the sedimentation equilibrium experiments the differential sedimentation technique gave similar results for the apoenzyme and enzyme to which different amounts of NAD were bound. Direct binding experiments using the photoelectric scanner of the ultracentrifuge showed that in the D_2O solutions the affinity of the apoenzyme for NAD decreased with the extent of binding. At the concentrations used for these experiments the first two NAD molecules were bound stoichiometrically. Binding at the other sites was substantially weaker with the fourth site showing the smallest affinity. The binding data, which are in agreement with those found earlier for aqueous systems, were used in conjunction with difference sedimentation studies to determine the change in the sedimentation coefficient accompanying the binding of NAD. The binding of four NAD molecules to the apoenzyme produced a change of about 4% in the sedimentation coefficient of the protein. Most of this change could be attributed to the contribution of the bound NAD to the buoyant weight of the complex. However, the corrected values indicated that there was a slight change in the sedimentation coefficient resulting from alterations in the frictional coefficient of the protein. Since there appeared to be no shift in the association–dissociation equilibria of the protein, these results may be interpreted in terms of conformational changes such as swelling or shrinkage (or change in shape) of the tetrameric protein molecules. Upon binding of four NAD molecules the tetramers contract by 1–2%. Although the results with one enzyme preparation suggest that binding of the first NAD molecule to the tetramers produced a different effect from that caused by subsequent binding, the changes were so small that definitive conclusions will have to await further studies.

Rabbit muscle glyceraldehyde phosphate dehydrogenase,[1] though consisting apparently of four identical polypeptide chains (Harris and Perham, 1965, 1968), has been found to have nonequivalent binding sites for the four NAD molecules. Each successive cofactor molecule is bound less strongly than the previous one (De Vijlder and Slater, 1968;

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[1] Abbreviations used are: GPDH, glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating)), EC 1.1.1.27; E, EN, EN_2, EN_3, and EN_4 refer to enzyme to which 0, 1, 2, 3, and 4 mol of NAD are bound, respectively.

Conway and Koshland, 1968; Velick *et al.*, 1971). This decrease in affinity, termed negative cooperativity (Conway and Koshland, 1968), has been interpreted in terms of subunit interactions with the binding of one NAD at one site inducing conformational changes in other subunits.

A variety of experimental techniques has been employed to demonstrate conformational changes in GPDH upon the addition of NAD. These include, for example, studies of the reactivity of SH groups (Conway and Koshland, 1968; Vas and Boross, 1970), of the optical rotatory dispersion (Listowsky *et al.*, 1965; Bolotina *et al.*, 1967) and of temperature-jump kinetics (Hammes *et al.*, 1971). In many cases the findings have been interpreted in alternative fashions (Fenselau, 1970a; De Vijlder and Harmsen, 1969; Velick *et al.*, 1971). Recently MacQuarrie and Bernhard (1971) demonstrated an intrinsic geometric nonequivalence in the active sites of the enzyme molecules and concluded that negative cooperativity is not induced.

In the light of these results, and the differences in interpretations, a study was undertaken to look for conformational changes in the enzyme as revealed by alterations in the hydrodynamic properties of the enzyme resulting from successive NAD binding. Hydrodynamic measurements have the advantage of being sensitive to bulk conformational changes in a protein, rather than simply reflecting local changes of individual amino acids or regions of amino acids (Kirschner and Schachman, 1971a).

Since hydrodynamic techniques are particularly sensitive to alterations in molecular weight of the protein under investigation (Smith *et al.*, 1973), experiments were performed to ascertain whether the observed changes in sedimentation coefficient were due to a small shift in the association–dissociation equilibrium involving the protein and its subunits or to shape or volume changes in the protein molecules. This problem is discussed further in the following paper (Smith *et al.*, 1973). Because GPDH samples were found to exist as subunits in addition to the predominant tetramer and since there were time-dependent changes in the molecular weight of the protein, the interpretation of the results from the difference sedimentation measurements was equivocal. Hence attempts were made to find conditions in which the enzyme was stabilized exclusively as tetramer. It was found that heavy water (D_2O) provided such a medium. Difference sedimentation studies performed in D_2O demonstrated that upon binding each NAD, the sedimentation coefficient of GPDH is altered. Most of the change was due to the increased buoyant weight of the complex caused by the bound NAD. However, some of the change upon the binding of four NAD molecules to the tetramers could be attributed to a 1–2% decrease in the hydrodynamic volume. Although these findings (Smith and Schachman, 1972) provide an experimental basis for the conclusion that the binding of each NAD molecule caused bulk conformational changes in the protein, we are unable to conclude that the binding of NAD at each site contributes equally to the change in sedimentation coefficient.

Experimental Section

Materials. Crystalline GPDH samples were obtained commercially from C. F. Boehringer (Mannheim Corp.). Solutions of the enzyme were prepared from the ammonium sulfate suspension by centrifuging in a clinical centrifuge, dissolving the precipitate in the appropriate buffer and then dialyzing against buffer. D_2O (99.88%) was obtained from Bio-Rad Laboratories and was stored in an airtight container in a refrigerator. NAD and DL-glyceraldehyde 3-phosphate (diethyl acetal, barium salt) were obtained from Sigma Chemical Co.

Methods. Enzyme assays were performed by the method of Velick (1955). The substrate, DL-glyceraldehyde 3-phosphate, was prepared from its diethyl acetal (barium salt) (Sigma Chemical Co., Technical Bulletin, No. 10). Experiments in aqueous buffers employed 0.1 M Tris-HCl–1 mM Na_2EDTA at pH 8, 9, and 10. The experiments in D_2O used 0.1 M Tris-HCl–1 mM Na_2EDTA (pD 8). The pD of D_2O solutions was taken as the pH meter reading plus 0.40 (Glasoe and Long, 1960). The pH or pD values refer to room temperature and would be slightly different in those experiments conducted at low temperatures due to the relatively high temperature coefficient for ionization of Tris.

Protein concentrations were determined either refractometrically in analytical ultracentrifuge experiments with a double-sector synthetic boundary cell (Schachman, 1957, 1963; Babul and Stellwagen, 1969) or spectrophotometrically. For the latter determinations specific absorbance coefficients at 280 nm of 1.00 $cm^2\ mg^{-1}$ for the holoenzyme and 0.829 $cm^2\ mg^{-1}$ for the apoenzyme were used (Fox and Dandliker, 1956b). Molar concentrations were calculated on the assumption of a molecular weight of 1.44×10^5 for tetrameric native GPDH (Harrington and Karr, 1965; Harris and Perham, 1968; Jaenicke *et al.*, 1968). ApoGPDH was prepared essentially by the method of Murdock and Koeppe (1964) except that the ratio of charcoal pulp to protein was doubled and the EDTA solution was adjusted to pH 8 with NaOH. The apoenzyme had a spectral ratio (A_{280}/A_{260}) of 2.0–2.1. After the charcoal treatment the enzyme was dialyzed into the appropriate buffer. Experiments in aqueous solutions were all performed at low temperatures because apoGPDH was found to be unstable at room temperature.

The concentration of NAD was determined spectrophotometrically with the molar extinction coefficient, $17.8 \times 10^3\ M^{-1}\ cm^{-1}$ at 260 nm (Winer, 1964).

All buffer densities were measured pycnometrically. Viscosity measurements were made with an Ostwald viscometer (Schachman, 1957).

Sedimentation Studies. All sedimentation studies were performed with a Spinco Model E analytical ultracentrifuge equipped with an electronic speed control and both schlieren and Rayleigh interference optical systems (Richards and Schachman, 1959). For the equilibrium and difference sedimentation experiments, double-sector cells with a 12-mm light path and sapphire windows were used. The optical system was focused at the two-thirds plane of the cell (Richards *et al.*, 1971).

The differential sedimentation experiments were conducted with two cup-type synthetic boundary cells (Pickels *et al.*, 1952) according to the mehod of Hersh and Schachman (1955). To allow simultaneous measurements on two different samples, we used two cells, one of which contained a 1° quartz wedge as the upper window, the other cell having a conventional quartz window with parallel surfaces. In this way one of the schlieren patterns was displaced vertically, allowing the boundary positions to be measured separately on a Gaertner microcomparator. Metallographic plates were used to record the schlieren patterns. In both cells, approximately 0.4 ml of the more concentrated solution was added to the main cell compartment and 0.24 ml was added to the cup. Boundary formation occurred at about 10,000

rpm, and during the layering, rotor acceleration was kept at a minimal level.

The sedimentation equilibrium experiments employed multichannel, carbon-filled, epoxy centerpieces (Yphantis, 1964). The initial protein concentrations were 0.5–1.0 g/l. Column heights of 3 mm were used routinely. The angular velocity was 20,000 rpm. Checks for the attainment of sedimentation equilibrium were made by photographing the patterns at the time calculated for equilibrium to be achieved and again after an interval of 10% of the calculated equilibrium time. The temperature was recorded with the RTIC unit but was maintained by the refrigeration unit operating alone since it has been found that the intermittent operation of the heating coil can cause convection (Gropper and Boyd, 1965). For several of the samples where results appeared not to be reproducible, the experiments were repeated after making the solutions 1% with respect to sucrose. The presence of this solute reduces the possibility of convection in the solution (Yphantis, 1964) and gave reassurance to our conclusion that nonreproducibility was due to the chemical system itself. No use was made of a layering fluid, such as fluorocarbon (FC-43) or silicone oil, to form a "false bottom," because of reports that these substances can cause protein aggregation at the solution–fluid interface (Adams and Lewis, 1968; Cassman and Schachman, 1971). Base lines were usually obtained by refilling the cell with water at the conclusion of the run and recentrifuging at the same temperature.

For computation of the data for the plots of ln c *vs.* r^2 and M_w *vs.* c, the methods of Teller *et al.* (1969) were used as applied by Hoagland and Teller (1969) to the analysis of GDPH samples.

Difference sedimentation experiments were performed by the method of Kirschner and Schachman (1971a,b) using double-sector cells with unfilled epoxy centerpieces or by using paired 2° single-sector cells as described by Gerhart and Schachman (1968) and by Schumaker and Adams (1968). The rotor speed was 60,000 rpm and the temperature was controlled with the RTIC unit. Because of the strong binding of NAD to GPDH, the concentration of unbound NAD in the experiments was usually very low. Hence changes in sedimentation coefficients resulting from differences in viscosity and density of the solutions were usually assumed to be insignificant, and no compensating solutes were added to the second solution. In cases where the concentration of free NAD was sufficient to alter significantly the viscosity and density of the solution the correction was calculated by measuring the viscosity and density of NAD solutions at higher concentrations and interpolating to the concentration region of interest.

All Rayleigh fringe patterns were photographed using spectroscopic IIG plates. Monochromatic light was produced with a Wratten 77A filter which, for location of the white-light fringe, was replaced with glass of the same thickness. The patterns were measured on a Nikon Model 6C microcomparator. Schlieren patterns were recorded on metallographic plates and measured on a Gaertner microcomparator.

Measurements of the binding of NAD to the enzyme were made with an ultracentrifuge equipped with a split-beam photoelectric scanning absorption optical system (Steinberg and Schachman, 1966). Various amounts of protein were added to a fixed concentration of NAD and the solutions were centrifuged at 60,000 rpm for 1.5 hr and scanned with light at 265 nm. The amount of NAD bound to the enzyme was then measured as the depletion of the 265-nm absorption in the NAD plateau compared with that of a solution of NAD alone. The pen deflection of the scanner was calibrated in terms of the absorption of an NAD solution as measured in a spectrophotometer. The absorption optical system was also employed to detect the presence of dissociated and aggregated species in solutions of apoGPDH. For this purpose a wavelength of 280 nm was used.

The sedimentation coefficient of NAD was measured in two ways. The first was to scan at intervals during sedimentation with light of 265 nm. The sedimentation coefficient was then calculated using the integrated form of the transport equation (Schachman, 1959). With this method the sedimentation coefficient was determined at an NAD concentration of 0.02 g/l. The second method involved forming a sharp boundary between buffer and a solution of NAD in the cup-type synthetic boundary cell (Pickels *et al.*, 1952) and photographing with schlieren optics in the usual way. The sedimentation coefficient was measured from the rate of movement of the position corresponding to the square root of the second moment of the gradient curves. This method allowed measurements to be made at an NAD concentration of 10 g/l. The results from the two methods agreed closely.

The partial specific volume, $\bar{V}$, of GPDH was assumed to be independent of the amount of bound NAD and was taken as 0.74 ml/g at 20° and 0.73 ml/g at 4–5° (Taylor and Lowry, 1956; Jaenicke *et al.*, 1968). In D_2O solutions the partial specific volume of the protein was taken as $\bar{V}/k$ where k, which accounts for exchangeable hydrogens, was assumed to be 1.0155 (Hvidt and Nielsen, 1966; Edelstein and Schachman, 1967). Similarly, in D_2O, protein molecular weights were assumed to be increased by the factor, k. The apparent specific volume of NAD was measured pycnometrically to be 0.56 ml/g in D_2O buffer and 0.57 ml/g in aqueous buffer.

Results

Difference Sedimentation in Aqueous Buffers. To investigate whether the sedimentation coefficient of apoGPDH is altered upon binding NAD, we performed experiments in which apoGPDH was placed in one side of the double-sector cell and in the other side was placed apoGPDH to which had been added different amounts of NAD per mole of enzyme. The observed $\Delta s/s_{av}$ values were corrected for the buoyant weight of bound NAD using the equation

$$\left(\frac{\Delta s}{s_{av}}\right)_{cor} = \frac{\Delta s}{s_{av}} - \frac{rM_L(1 - \bar{V}_L\rho)}{M_P(1 - \bar{V}_P\rho) + r(M_L/2)(1 - \bar{V}_L\rho)} \quad (1)$$

where s_{av} is the average of the two weight-average sedimentation coefficients and Δs is the difference between the two weight-average sedimentation coefficients, and M_L and $\bar{V}_L$, $\bar{M}_P$ and $\bar{V}_P$ are the molecular weight and partial specific volume of ligand (NAD) and protein, respectively, ρ is the density of solvent and r is the number of moles of NAD bound per mole of protein (Klotz, 1953). For computation of the corrections, the binding of the first and second moles of NAD per mole of GPDH to give the complexes, EN and EN_2, was assumed to be stoichiometric (Conway and Koshland, 1968; De Vijlder and Slater, 1968). For binding beyond the EN_2 stage the free NAD in solution was calculated by solving eq 2, where [N] is the free NAD concentration, [E_t] and [N_t]

$$[N]^3 + [N]^2(4[E_t] + K_4 - [N_t]) + [N](K_3K_4 + 3K_4[E_t] - K_4[N_t]) + 2K_3K_4[E_t] - K_3K_4[N_t] = 0 \quad (2)$$

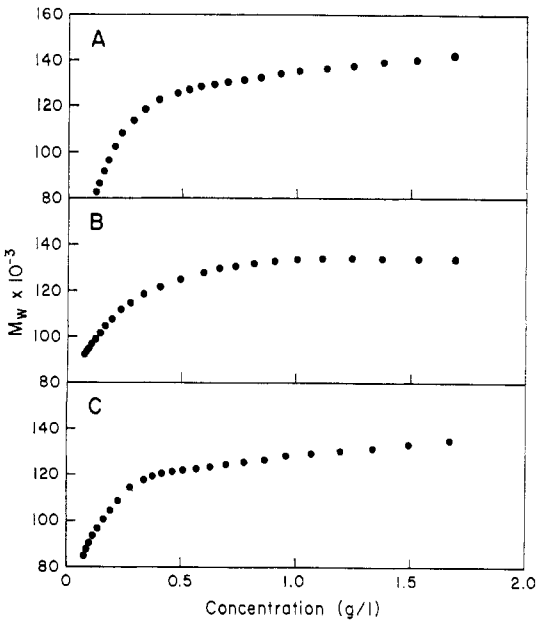

FIGURE 1: Sedimentation equilibrium of apoGPDH (A) and apoGPDH to which 1 mol of NAD (B) and 2 mol of NAD (C) were added per mole of protein. The solutions were in 0.1 M Tris–1 mM EDTA (pH 8). Sedimentation was performed at 20,000 rpm and 7° and the calculations refer to photographs taken 18 hr after sedimentation was commenced. The initial protein concentration of each sample was 0.85 g/l. The results are plotted as weight-average molecular weight (M_w) calculated with a computer (see Methods) as a function of protein concentration in grams per liter.

are the total concentrations of enzyme and NAD added to the solution, and K_3 and K_4 are the dissociation constants for the third and fourth sites, respectively. From the computed values of [N], the corresponding r values were calculated. The values of $(\Delta s/s_{av})_{cor}$ were determined for the binding constants of both Conway and Koshland (1968), $K_3 = 3 \times 10^{-7}$ M and $K_4 = 2.6 \times 10^{-5}$ M, and De Vijlder and Slater (1968), $K_3 = 4 \times 10^{-6}$ M and $K_4 = 3.5 \times 10^{-5}$ M. As seen in Table I the binding of almost 3 mol of NAD per mol of apoGPDH caused changes of 1.91 or 1.97% in sedimentation coefficient depending on which constants were used for the corrections. These changes must reflect structural differences in the protein itself as a result of NAD binding. A control experiment with identical solutions of native GPDH in each side of the cell gave a value of +0.04% (+0.003 S), the departure from zero reflecting the limits of precision in the technique. Table I also shows the changes in sedimentation coefficient stemming from different extents of binding of NAD. The largest value of $(\Delta s/s_{av})_{cor}$ was obtained upon the binding of the first NAD molecule to apoGPDH; subsequent NAD binding led to smaller increments.

Effect of NAD on Molecular Weight of Protein. Because difference sedimentation experiments do not distinguish between shifts in association–dissociation equilibria and changes in shape or volume of the protein molecules, sedimentation equilibrium experiments were initiated in order to determine whether NAD binding altered the weight-average molecular weight of apoGPDH at pH 8. Such samples, when observed at concentrations of about 8 g/l. within 2–3 days after the charcoal treatment, showed single boundaries (without evident dissociation) in sedimentation velocity experiments. In high-speed sedimentation equilibrium studies (Yphantis, 1964) at much lower protein concentrations, however, nonlinear plots of log c *vs.* r^2 were

TABLE I: Effect of NAD on the Sedimentation Coefficient of GPDH.[a]

Expt	Samples[b]	$\Delta s/s_{av}$[c] (%)	Δs (S)	$(\Delta s/s_{av})_{cor}$[d] (%)
1	GPDH *vs.* GPDH	+0.04	+0.003	+0.04
2	E *vs.* EN_3	+3.81	+0.27	+1.91,[e] 1.97[f]
3	E *vs.* EN	+1.61	+0.12	+0.96
4	EN *vs.* EN_2	+1.04	+0.075	+0.40
5	EN_2 *vs.* EN_3	+0.62	+0.045	+0,[e] 0.06[f]

[a] All experiments were performed at 7.7° in 0.1 M Tris-HCl–1 mM EDTA buffer at pH 8. The protein concentration in each case was 8.46 g/l. [b] GPDH refers to enzyme prepared from the commercially available sample by dialysis against buffer. ApoGPDH (E) was prepared as described in the Methods section. The binding of the first (EN) and second (EN_2) moles of NAD per mole of GPDH was assumed to be stoichiometric (Conway and Koshland, 1968; De Vijlder and Slater, 1968). When EN_3 is formed by the addition of 3 mol of NAD/mol of enzyme, the third NAD was calculated to be 96% bound if the binding constants of Conway and Koshland (1968) were used, and 86% bound if the binding constants of De Vijlder and Slater (1968) were used. [c] The values $\Delta s/s_{av}$ are the observed percent changes in sedimentation coefficient. No corrections were made for contributions of unbound NAD to the viscosity and density of the solution because the first 3 mol of NAD are virtually stoichiometrically bound to the enzyme. [d] Corrections for the buoyant weight of bound NAD were made according to eq 1 (see Kirschner and Schachman, 1971a,b). [e] This value was calculated with the binding constants of Conway and Koshland (1968). [f] This value was calculated with the binding constants of De Vijlder and Slater (1968).

obtained indicating dissociation of the protein into subunits. Figure 1 gives the results of these experiments in terms of the weight-average molecular weight, M_w, as a function of concentration. Since the object of these experiments was to determine whether *differences* in molecular weight distributions as a function of added NAD could be the basis for the observed changes in sedimentation coefficients, no attempts were made to obtain a precise curve-fitting of the data. Although the presence of monomeric subunits is indicated by Figure 1, the results were not of sufficient reproducibility to permit determination of unique values of a pair of equilibrium constants such as would describe a monomer–dimer–tetramer system. Furthermore, as indicated later, charcoal-treated GPDH appears to undergo slow time-dependent losses of activity indicative of slow irreversible changes in the quaternary structure of the protein. Such observations have been reported by Hoagland and Teller (1969) whose preparations appear to have been even less stable. The results of a number of sedimentation equilibrium experiments indicated also traces of aggregates which made unique curve-fitting difficult. It is clear from experiments such as those illustrated in Figure 1 that species of different molecular weight do exist in samples of the charcoal-treated enzyme. Hence, although the experiments were not sufficiently accurate nor reproducible to conclude that NAD binding caused differences in the degree of association, such an interpretation is possible.

Effect of pH on Dissociation of GPDH. Although the re-

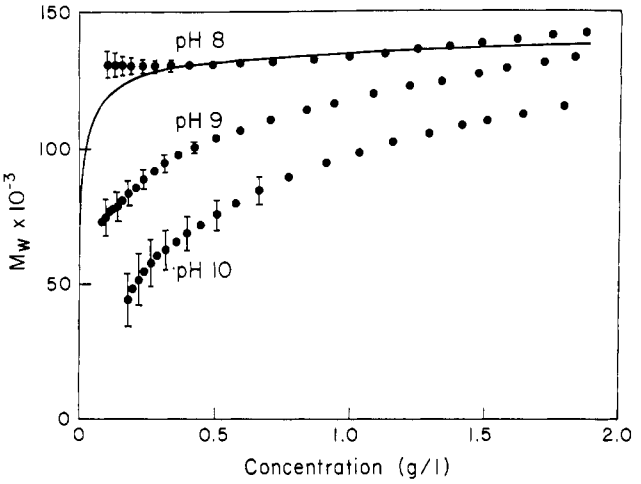

FIGURE 2: Weight-average molecular weight (M_w) *vs.* concentration of GPDH at different pH values. All solutions were in 0.1 M Tris–1 mM EDTA and the pH values were adjusted at room temperature. Sedimentation was performed at 20,000 rpm and 7° for 18 hr. The initial protein concentration of each sample was 0.80 g/l.

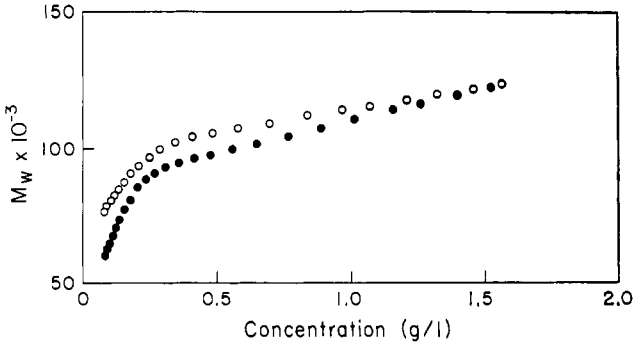

FIGURE 3: The effect of an excess of NAD on the weight-average molecular weight (M_w) of GPDH at pH 9. The symbol, ●, refers to apoGPDH, and ○ refers to apoGPDH to which a 100-fold molar excess of NAD had been added. The buffer contained 0.1 M Tris–1 mM EDTA and the initial protein concentration was 0.97 g/l. Sedimentation was performed at 20,000 rpm and the photographs used for the calculations were taken after 18 hr of sedimentation. Protein concentration in grams per liter is shown on the abscissa.

sults of the sedimentation equilibrium experiments did not clarify the effect of NAD binding on the association–dissociation equilibria of GPDH, it seemed likely that studies at different pH values might be more discriminating. Figure 2 shows the effect of pH on the dissociation of GPDH for which no special attempts were made to remove NAD. As the pH was increased above pH 8 the degree of dissociation also increased. The solid line in Figure 2 represents the theoretical weight-average molecular weight distribution for a dimer–tetramer equilibrium system with an association constant of 55 l./g, the value reported for similar conditions by Hoagland and Teller (1969). This value reasonably approximates the pH 8 data, although a slight amount of aggregated material was also evident (*cf.* Hoagland and Teller, 1969). At pH values above 8 the position of equilibrium was shifted in favor of the subunits and the presence of monomers was indicated.

Attempts to Perturb the Association–Dissociation Equilibrium of GPDH by NAD at pH 9. Since the extent of dissociation of GPDH was greater at pH 9 than at pH 8, any perturbation of an existing equilibrium by NAD would be expected to be more easily detected at the former pH, because the weight-average molecular weight varies more markedly with concentration in the experimentally accessible range (Figure 2). Hence a solution of apoGPDH was prepared and dialyzed against a Tris buffer at pH 9. To one aliquot was added a 100-fold molar excess of NAD; a second aliquot was diluted to the same concentration with buffer, and both samples were submitted to equilibrium centrifugation simultaneously. The results, illustrated in Figure 3, show that NAD caused an increase in the degree of association of the protein. Thus these results, together with those for the pH 8 solutions, indicate that molecular weight changes may be partly or wholly responsible for the changes in sedimentation coefficient stemming from NAD binding. However, the sedimentation equilibrium data were not sufficiently accurate, nor were the association–dissociation equilibrium of GPDH sufficiently well defined, to warrant definitive conclusions as to the cause of the change in sedimentation coefficient accompanying NAD binding.

Effect of D_2O on Stability and Molecular Weight of GPDH. When aqueous solutions of apoGPDH were maintained at room temperature, precipitation of protein was invariably observed, with the time for visible flocculation depending on the protein concentration. At low concentrations (less than 1 g/l.) visible precipitation was not observed for extended periods, but at concentrations in the range 5–10 g/l., precipitation was observed within hours after charcoal treatment. At low temperatures (0–4°) precipitation was delayed significantly, but nevertheless turbidity was observed in 3–5 days with solutions of 5–10 g/l. Addition of NAD greatly stabilized the protein. Similar observations have been reported by other workers, some of whom have reported a time-dependent loss of specific activity of the enzyme (Velick and Furfine, 1963; Velick *et al.*, 1953; Colowick *et al.*, 1966). The inability to describe more precisely the GPDH systems used in the preceding experiments may be due in part to time-dependent structural alterations in the enzyme, including changes in molecular weight. Hence attempts were initiated to find conditions leading to stable apoenzyme preparations. Preliminary experiments showed that when buffers were made up in heavy water (D_2O) instead of ordinary water (H_2O), the tendency of the apoenzyme to precipitate was dramatically abolished. At both room temperature and low temperature, apoGPDH solutions in this medium were found to be stable for weeks. A sample stored at low temperature for several weeks exhibited an apparently homogeneous sedimenting boundary as observed by schlieren optics. Hence experiments were initiated to study the effect of D_2O on the enzymic activity and molecular weight of the protein.

ApoGPDH was prepared in the normal way and separate aliquots were dialyzed against H_2O and D_2O (buffer salts were present in each case). Solutions were prepared at two different concentrations (9.1 and 0.091 g per l.) and portions were incubated at room temperature (20–25°). Aliquots were then assayed at intervals over a period of 6 days following preparation of the samples. The results, illustrated in Figure 4, show enzyme activities expressed in terms of the percentage of the specific activity observed on the first day after preparation of the solutions. It is seen that the samples in D_2O at both protein concentrations retained full activity over the whole period. The sample of apoGPDH in H_2O at 9.1 g/l. gave heavy flocculation within hours of preparation and was not assayed. At the lower concentration (0.091 g/l.) in H_2O no precipitation was observed but considerable inactivation occurred over the time period of the experiment.

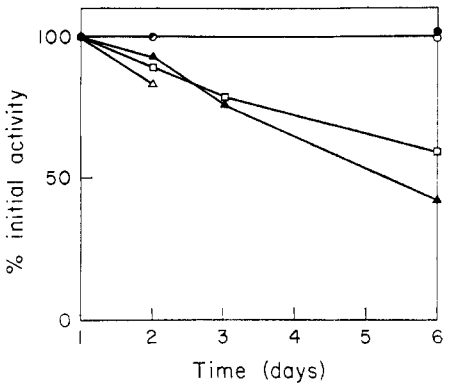

FIGURE 4: Stabilization of apoGPDH by D_2O. Samples were prepared in 0.1 M Tris–1 mM EDTA at pH or pD 8 and the solutions were placed under the following conditions of protein concentration and temperature: 9.1 g/l., 20–25°, in D_2O (●); 0.091 g/l., 20–25°, in D_2O (○); 0.091 g/l., 20–25°, in H_2O (□); 9.1 g/l., 0–4°, in H_2O (△); 0.091 g/l., 0–4°, in H_2O (▲). The per cent initial activity (on the ordinate) was calculated relative to the activity of each sample as assayed on the first day after preparation of the sample, and the time in days (on the abscissa) refers to the time after this first assay. The samples were diluted appropriately into regular assay buffer (0.03 M sodium pyrophosphate, pH 8.4) and assayed in the usual way (see Methods).

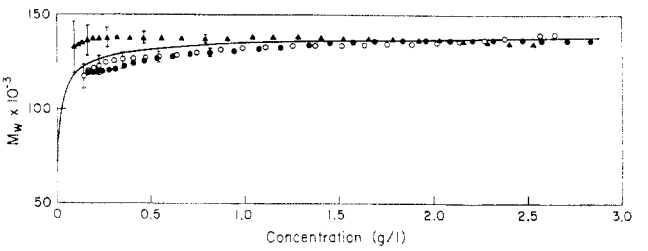

FIGURE 5: Sedimentation equilibrium in D_2O buffer of apoGPDH (●) and apoGPDH to which a 1-fold (○) and 10-fold (▲) molar excess of NAD had been added. The solvent for all experiments was 0.1 M Tris–1 mM EDTA at pD 8. Sedimentation equilibrium experiments were performed at 20,000 rpm and 4° with a multicompartment cell containing three pairs of rectangular openings for the three solutions and their respective reference solvents. The initial protein concentration of each sample was 0.87 g/l. The weight-average molecular weight (M_w), on the ordinate, as a function of c in grams per liter (on the abscissa) was calculated as described in Methods.

Preparations of apoGPDH in H_2O buffer also were incubated at low temperature (Figure 4). At 9.1 g/l. samples lost an appreciable amount of activity during the first 2 days and on day 3 visible aggregation was observed; hence the assays were discontinued after this time. At 0.091 g/l., where no turbidity was observed, appreciable loss of activity was found.

All of the experiments described above involved assaying the samples in aqueous buffer after diluting the samples from the respective media in which they had been incubated. In the regular assay buffer, enzyme samples diluted from D_2O buffer had essentially the same initial specific activity as samples diluted from aqueous buffers. When assays were performed directly in the D_2O buffer itself (0.1 M Tris–1 mM EDTA, pD 8) and compared with a corresponding sample in H_2O buffer, it was found that the specific activity in D_2O was approximately 50% of that in H_2O, presumably due to a specific isotope effect on the enzymic mechanism.

To correlate the observed loss of enzymic activity of aqueous solutions of apoGPDH with molecular weight changes, we prepared an aqueous solution of the apoenzyme and submitted it to ultracentrifugation at 60,000 rpm in a centrifuge equipped with a photoelectric scanning system. The protein was at a concentration of 0.8 g/l. and scans at 280 nm were made on 4 successive days. In each case the bulk of the protein sedimented at approximately $s_{20,w} = 7.4$ S. However slight amounts of more slowly sedimenting species and also of more aggregated species were observed and the proportion of these increased with time. Presumably the production of these dissociated and aggregated species was responsible for the inactivation of apoGPDH solutions and resulted in visible precipitation of the more concentrated solutions. Apparently it was these different molecular weight species which were observed in sedimentation equilibrium experiments such as those illustrated in Figures 1–3. Such solutions clearly were not in chemical equilibria in terms of oligomers and subunits.

In view of the marked stabilization of the apoenzyme by D_2O, a further experiment was performed to assess the effect of D_2O on freezing and thawing the enzyme. It was found that whereas aqueous solutions of apoGPDH at a concentration of 9.1 g/l. precipitated heavily when frozen and thawed, corresponding solutions in D_2O showed total absence of precipitation when treated in the same way. Furthermore, such samples retained most of their original specific activity.

The effect of D_2O on the stability and activity of apoGPDH was so striking that it seemed likely that D_2O might also influence the degree of association of the enzyme. Accordingly sedimentation equilibrium experiments at room temperature (23.9°) were performed on D_2O solutions of apoGPDH (E) and apoGPDH to which 1 (EN) and 10 (predominantly EN_4) moles of NAD were added per mole of enzyme. The plots of log c *vs.* r^2, where c is the concentration measured as fringe displacement and r is the distance from the axis of rotation, were linear over a concentration range from 0.1 to 3 g per l. This indicated that the principal species had neither dissociated nor aggregated significantly. A molecular weight of 140,000 was calculated for each solution, a result in reasonable agreement with the value, 146,000, expected for fully deuterated enzyme. These results show clearly that D_2O stabilizes the enzyme as tetramers at room temperature, irrespective of the presence or absence of the cofactor, NAD. Figure 5 shows the results of an analogous sedimentation equilibrium experiment at low temperature (4°) on the same solutions used for the room temperature experiments. At the lower temperature some slight dissociation was observed. It should be noted, however, that this dissociation was reversible since all samples in D_2O, including those sedimented at room temperature, were stored in a refrigerator before the experiments were performed. Furthermore the enzyme was stabilized against precipitation at low temperatures, in contrast with aqueous solutions of the apoenzyme. The solid line of Figure 5 represents a dimer–tetramer system with an association constant of 55 l./g and it is seen that the data are reasonably approximated by it. The data are not of sufficient accuracy to warrant conclusions as to the effect, if any, of NAD on the association–dissociation equilibria.

Binding of NAD to GPDH in D_2O Buffers. Because of the considerable effect of D_2O on the physical properties of GPDH it was of importance to measure the binding of NAD in this environment. Various amounts of apoGPDH were added to a fixed concentration of NAD (4.38×10^{-5} M) and the solutions were sedimented at 21° in an analytical ultracentrifuge and scanned after 90 min of sedimentation with light of 265 nm. The amount of NAD bound to the enzyme

(r) was then calculated from the depletion of the 265-nm absorption in the plateau region containing only NAD.

Since the binding of NAD to GPDH caused an alteration in its sedimentation coefficient (see below), the amount of bound NAD calculated from measurements of the depletion of NAD behind the GPDH boundary is subject to potential error (Steinberg and Schachman, 1966). Hence experiments were conducted to determine whether this error was significant; for this purpose it was necessary to measure the sedimentation coefficient of NAD in the D_2O buffer employed for the binding experiments. This value was found to be 0.39 S at 21° ($s_{20,w}$ = 0.52 S). The concentration of free NAD, measured as described above from the concentration in the NAD plateau region, was then used in

$$[A] = [A_0]\frac{s_P - s}{s_P - s_A} \quad (3)$$

to calculate s_A, where s_A and s_P are the weight-average sedimentation coefficients of NAD and the enzyme, respectively, s_A is the sedimentation coefficient of free NAD, and $[A_0]$ is the initial concentration of NAD (Steinberg and Schachman, 1966). The value of s_P was calculated for the concentration of protein used in each particular experiment from the known dependence of the sedimentation coefficient on concentration (see next section). The calculated value of s_A was then used in

$$[A] = \frac{[A_0]\left\{s_P - s_A - \frac{1}{n}(s_P - s_P)\right\} + [P_0](s_P - s_P)}{s_P - s_A - \frac{1}{n}(s_P - s_P)} \quad (4)$$

to recalculate[2] [A]. For the use of eq 4 (Steinberg and Schachman, 1966), $s_P - s_P$ values were measured as described in the final section of this paper; $[P_0]$ is the total protein concentration. In these calculations, the dissociation constants for the first two binding sites were assumed to be negligible and hence the calculations were made only for those values of r greater than 2. Hence n was assumed to be 2, a reasonable assumption since binding of the third and fourth NAD molecules contributed equally to the increment in sedimentation coefficient, $s_P - s_P$. From the close correspondence of the measured and calculated values of [A] it was concluded that corrections to the binding data caused by the change in sedimentation coefficient of the protein were negligible. The binding curve is shown in Figure 6. The results are consistent with the conclusion that the first two NAD sites are occupied stoichiometrically (very low dissociation constants) and the dissociation constants for the third (K_3) and fourth (K_4) sites are approximately 5×10^{-7} and 1×10^{-5} M, respectively. The values of K_3 and K_4 were computed by curve fitting of the data shown in Figure 6, This was accomplished by using eq 2 and $r = ([N_t] - [N])/[E_t]$. This calculation

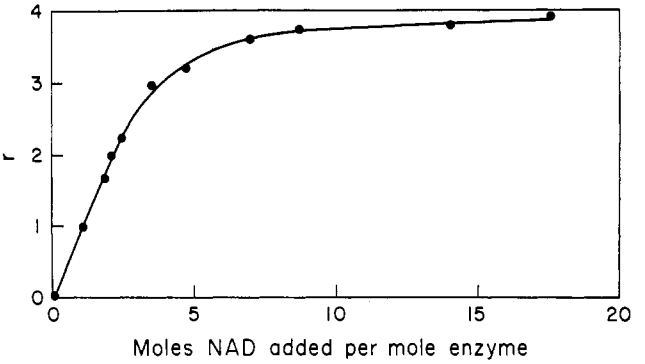

FIGURE 6: The binding of NAD to apo-GPDH in D_2O. The buffer contained 0.1 M Tris–1 mM EDTA at pD 8. Solutions were prepared by adding the various amounts of apoGPDH to a fixed concentration of NAD (4.38×10^{-5} M) and the solutions were then sedimented at 21.4° in an analytical ultracentrifuge equipped with a split-beam photoelectric scanning absorption optical system. After 90 min of sedimentation the solution was scanned at 265 nm and the concentration of unbound NAD was measured from the pen deflection in the plateau region representing only NAD. On the ordinate is r, the number of moles of NAD bound per mole of enzyme, and the abscissa gives the total amount of NAD (in moles) per mole of enzyme.

indicates that K_4 is significantly greater than K_3. Thus the negative cooperativity of binding pertains in D_2O solutions as reported previously for aqueous solutions (De Vijlder and Slater, 1968; Conway and Koshland, 1968).

Concentration Dependence of Sedimentation Coefficient of GPDH in D_2O. Since the sedimentation equilibrium experiments in D_2O buffers failed to detect dissociation of apoGPDH or GPDH to which NAD had been added, it seemed unlikely that NAD binding had any influence on the degree of association of the enzyme in this medium. Nonetheless it was decided to test this conclusion further by subjecting the samples to differential sedimentation, which is sensitive to small changes in association–dissociation equilibria (Smith *et al.*, 1973).

Figure 7 shows representative schlieren patterns from a differential sedimentation experiment. In each case protein at a concentration of 4.35 g/l. was layered over a similar solution at 8.7 g/l. The lower pattern represents apoGPDH (E) and the upper pattern represents EN. A similar experiment was performed with EN_4. In each case the radial positions of both the upper (integral) boundary and the differential boundary were measured as a function of time. Plots of the logarithms of these positions *vs.* time were linear, and the slopes gave values of the sedimentation coefficient, s_1, of the protein in the upper solution at concentration c_1 and the rate of movement, s_D, of the differential boundary (Hersh and Schachman, 1955). From the values of s_1 and s_D the sedimentation coefficient at infinite dilution, s_0, and k_{app} were determined as shown in Table II. Since the values of k_{app} were calculated from the parameters, $\Delta s/\Delta c$ and s_0, any scatter in the results from the different experiments is due to scatter in the value of s_0; $\Delta s/\Delta c$ is measured directly from the movement of the integral and differential boundaries within the same experiment and therefore is not susceptible to slight changes in temperature and rotor speed which contribute generally to imprecision in sedimentation velocity experiments.

As seen in Table II the values of k_{app} are very similar for E, EN, and EN_4. The slight increases in s_0 for EN and EN_4 relative to E are attributed principally to the increase in molecular weight of the sedimenting units due to the bound

[2] In principle [A] should be calculated directly from eq 4, rather than from eq 3. The use of eq 4, however, requires measurements of $\bar{s}_A$ which ordinarily are obtained from the patterns produced by the photoelectric scanner. For many of the samples the protein concentration was so high that the optical density in the plateau region containing protein was greater than that measurable by the scanner. Hence, eq 3 was used to obtain an approximate value of $\bar{s}_A$ which was then used in eq 4 for an evaluation of the magnitude of the potential error stemming from the change in the sedimentation coefficient of the protein accompanying NAD binding (Steinberg and Schachman, 1966).

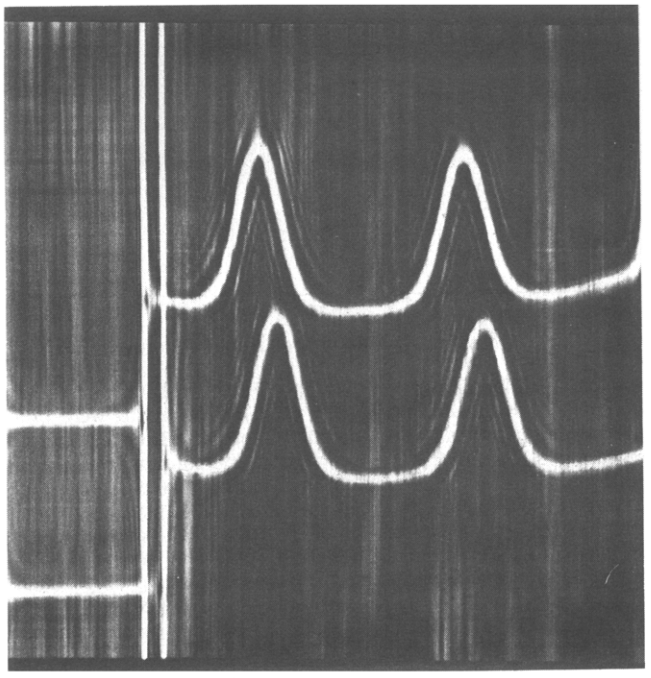

FIGURE 7: Schlieren patterns from differential sedimentation experiments. The effect of NAD on the concentration dependence of the sedimentation coefficient of apoGPDH in D_2O (0.1 M Tris–1 mM EDTA, pD 8) was investigated. The lower pattern represents apoGPDH and the upper pattern represents apoGPDH to which a 1-fold molar excess of NAD was added. In each case protein at a concentration of 4.35 g/l. was layered over a corresponding protein solution at 8.7 g/l. in cup-type synthetic boundary cells, one of which had a negative wedged window. The temperature was 19° and the rotor speed 60,000 rpm. Sedimentation is from left to right and the above patterns were obtained approximately 30 min after reaching speed.

NAD (see next section). If there had been a substantial shift in the association–dissociation equilibrium involving the tetramers and subunits the values of k_{app} would have decreased markedly (Kirschner and Schachman, 1971b; Smith *et al.*, 1973). Thus the observation that k_{app} was not affected significantly by NAD binding provides confirmation of the sedimentation equilibrium experiments which showed no change in the state of association of apoGPDH in D_2O upon the addition of NAD. The slight decrease in k_{app} for EN_4 (compared to E or EN) may be due to an increased compactness of the sedimenting unit which leads to a lowered frictional factor. There also may be a small experimental error since the value of s_0 for EN_4 (relative to E and EN) is greater than that measured by the difference sedimentation method (next section). It should be noted that E and EN were measured in the same sedimentation experiment with wedged windows and EN_4 was examined in a separate experiment; hence the slight difference between the two sets of experiments may be in part due to errors in s_0 as mentioned above.

Difference Sedimentation in D_2O Buffers. In view of the very marked stabilization of the enzyme by D_2O and the evidence that dissociation and association of the tetramers were markedly suppressed in this solvent, difference sedimentation studies were initiated with some assurance that changes in sedimentation coefficient resulting from NAD binding (besides those due to the increase in buoyant weight of the protein molecules) are in fact due to shape or volume changes in the tetramer itself.

Table III summarizes the results of a series of experiments on one enzyme preparation in which the sedimentation coefficient of apoenzyme was compared with those of the enzyme containing various amounts of NAD. Columns 2 and 3 show the molar concentrations of enzyme, $[E_t]$, and NAD, $[N_t]$, used in the respective experiments and the fourth column gives the number of moles of NAD bound per mole of enzyme, r. This value was calculated from the values of the binding constants as determined above, with the aid of eq 2. The observed values of the percent change in sedimentation coefficient, $(\Delta s/s_{av})_{obsd}$, are shown in column 5.

TABLE II: Effect of NAD Binding on the Concentration Dependence of the Sedimentation Coefficient of GPDH in D_2O.

Sample[a]	s_1[b] (S)	s_D[b] (S)	s_0[c] (S)	k_{app}[c] (l./g)
E	7.02	6.50	7.28	0.0082
EN	7.08	6.55	7.34	0.0082
EN_4	7.32	6.81	7.58	0.0078

[a] All samples were in 0.1 M Tris–1 mM EDTA (pD 8). The samples E, EN, and EN_4 refer to apoGPDH and apoGPDH to which a 1-fold and 10-fold molar excess of NAD were added, respectively. [b] The coefficients s_1 and s_D were obtained in differential experiments and refer to the rates of movement of the integral and differential boundaries, respectively. In each case protein at a concentration of $c_1 = 4.35$ g/l. was layered on a corresponding sample at $c_2 = 8.7$ g/l. [c] The values s_0 and k_{app} were obtained by assuming that the sedimentation coefficients of the protein samples are described by the equation $s = s_0(1 - k_{app}c)$, where s_0 is the sedimentation coefficient at infinite dilution and s is that at concentration c. The parameter k_{app} is defined as $-(1/s_0)(\Delta s/\Delta c)$. As shown by Hersh and Schachman (1955) $\Delta s/\Delta c$ is obtained from the relationship $s_D = s_1 + c_2(\Delta s/\Delta c)$ and hence s_0 and k_{app} may be obtained readily.

Because of the strong binding of NAD to GPDH no inert solute was added to the apoenzyme solution to compensate for the density and viscosity of unbound NAD in the other solution. For molar ratios of NAD to enzyme of less than 2:1 the concentration of unbound NAD is negligible (Figure 6). To allow corrections to be made for cases where the free NAD concentration was not negligible, we measured the viscosities (η) of NAD solutions of different concentration relative to the viscosity of buffer (η_0) and interpolated to the concentration of interest using the equation

$$\eta/\eta_0 = 1 + 0.00315c \qquad (5)$$

where c is the NAD concentration (g/l.) The corresponding solution density (ρ) was calculated from

$$\rho = \rho_0 + 0.001c(1 - \bar{V}\rho_0) \qquad (6)$$

where ρ_0 is the solvent density and $\bar{V}$ is the apparent specific volume which was measured pycnometrically and found to be 0.56 ml/g in the D_2O buffer. From these values the $(\Delta s/s_{av})_{obsd}$ values were corrected for the viscosity and density due to free NAD (Schachman, 1957). The molar concentration of free NAD, [N], was calculated from the known total concentrations of enzyme and NAD according to eq 2 with the values of K_3 and K_4, 5×10^{-7} and 1×10^{-5} M, respectively, The same value of [N] was used to calculate the number of

TABLE III: Effect of NAD on the Sedimentation Coefficient of GPDH in D_2O Buffer.[a]

Expt	$[E_t] \times 10^5$	$[N_t] \times 10^5$	r	$\Delta s/s_{av}$[b] (%)	$\Delta s/s_{av}$[c] (%)	Δs[d] (S)
1	5.99	1.50	0.25	0.00	0.00	0.000
2	5.52	2.76	0.50	0.23	0.23	0.012
3	5.99	4.49	0.75	0.53	0.53	0.028
4	6.05	6.05	1.00	0.59	0.59	0.031
5	5.52	8.28	1.50	1.05	1.05	0.055
6	6.05	12.1	2.00	1.77	1.77	0.10
7	5.52	13.8	2.49	2.11	2.11	0.11
8	6.05	18.1	2.97	2.77	2.77	0.15
9	2.76	13.8	3.77	3.56	3.61	0.19
10	5.52	27.6	3.86	4.06	4.15	0.22
11	5.99	30.0	3.87	3.37	3.46	0.18
12	6.05	60.5	3.97	3.73	4.15	0.22
13	3.00	59.9	3.98	3.19	3.75	0.20

[a] The results of difference sedimentation experiments in which apoGPDH was run against an identical concentration ($[E_t]$) of apoGPDH to which various amounts of NAD were added to give a total molar NAD concentration of $[N_t]$. The number of moles of NAD bound per mole of protein, r, was calculated from the measured binding constants as described in the text. All experiments were performed at 20–24° and solutions were centrifuged at 60,000 rpm. The buffer was 0.1 M Tris–1 mM EDTA (pD 8). [b] The observed per cent differences in sedimentation coefficient. [c] The observed % differences in sedimentation coefficient were corrected for the contribution of unbound NAD to the viscosity and density of the solvent as described in the text. [d] The absolute magnitude of the difference in sedimentation coefficient.

moles of NAD bound per mole of enzyme (r). These values, $\Delta s/s_{av}$, are presented in column 6 and the corresponding absolute change in weight-average sedimentation coefficient, Δs, is shown in column 7. As an additional check that the changes in s do not represent a shift in the association–dissociation equilibria, two of the experiments (9 and 13, Table III) were performed with protein at an appreciably lower concentration than used in the other experiments. It is clear that the values of $\Delta s/s_{av}$ were independent of protein concentration.

The results of the difference sedimentation experiments are summarized in Figure 8 as a plot of $\Delta s/s_{av}$ *vs.* r. We found what appeared to be slight systematic differences between two different samples of enzyme; hence the values obtained with these preparations have been denoted by different symbols. With a third preparation of enzyme even greater variations were found but these were shown to be correlated with NAD-dependent differences in the concentration dependence of the sedimentation coefficient of the enzyme and with evident dissociation of the protein as revealed by sedimentation equilibrium experiments (Yphantis, 1964). Thus results for this preparation are not included. All of the results presented in Figure 8 pertain to preparations having k_{app} values given in Table II. Since much of the observed increase in the sedimentation coefficient of the protein upon binding NAD is attributable to the added buoyant weight of the ligand, corrections were made according to eq 1. These corrected values, $(\Delta s/s)_{cor}$, are also shown in Figure 8.

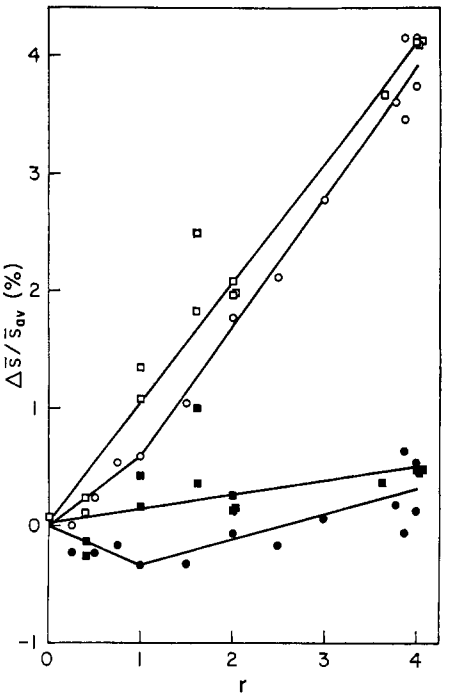

FIGURE 8: The effect of NAD binding on the sedimentation coefficient of GPDH in D_2O buffer containing 0.1 M Tris–1 mM EDTA (pD 8) at temperatures of 20–24°. The ordinate gives $\Delta\bar{s}/\bar{s}_{av}$ in per cent and the abscissa presents r, the moles of NAD bound per mole of enzyme, calculated as described in the text. The empty points (○ for preparation 1 and □ for preparation 2) represent the percent differences in sedimentation coefficient ($\Delta\bar{s}/\bar{s}_{av}$) between apoGPDH and the protein to which the various amounts of NAD were bound. The text presents details of the calculations required to compensate for the viscosity and density due to the unbound NAD. The observed values of $\Delta\bar{s}/\bar{s}_{av}$ were corrected for the buoyant weight of bound NAD to give $(\Delta\bar{s}/\bar{s}_{av})_{cor}$ represented by ● and ■. These values in percent are also represented by the ordinate. All sedimentation experiments were performed at 60,000 rpm. Experiments with preparation 1 were performed by the difference sedimentation technique based on interference optics (Kirschner and Schachman, 1971a). Those with preparation 2 were performed with paired cells, one containing plane windows and the other having one wedged cell window.

In making these corrections we assumed that nine of the hydrogen atoms on each NAD molecule were exchangeable and had been replaced by deuterium atoms.

Clearly the major part of the increase in sedimentation coefficient is due to the increase in buoyant weight of the sedimentating protein. The corrected values indicate that the binding of four moles of NAD per mole of enzyme results in a slight net increase in the sedimentation coefficient of 0.3–0.6% depending on the preparation. With one sample it appeared that the binding of the first NAD molecule to the apoenzyme caused a decrease in the sedimentation coefficient and that subsequent binding to the other sites in the tetramers led to an increase in the sedimentation coefficient. This conclusion requires a disproportionate emphasis being placed on the origin ($r = 0$), but this seems warranted because of the precision of the difference sedimentation technique (Kirschner and Schachman, 1971a). The data obtained with the second preparation of enzyme differ in showing that the binding of each NAD molecule leads to a very slight increase in sedimentation coefficient.

The scatter in the values given in Figure 8 for values of r greater than 3 represents to a large extent the uncertainty in the values of the binding constants as determined from

Figure 6. Any errors in these constants are reflected as errors in the calculated concentration of unbound NAD and hence of the corrections for the viscosity and density. In addition, of course, there would be a corresponding uncertainty in the values of r.

Discussion

A basic aim of the present work was to determine whether the binding of NAD to GPDH produced changes in the hydrodynamic properties of the enzyme and to interpret these changes in terms of its structure and function.

Difference sedimentation experiments (Table I), conducted in aqueous buffers similar to those used by workers who had reported that the enzyme exhibited negative cooperativity in its binding of NAD (De Vijlder and Slater, 1968; Conway and Koshland, 1968), did show that the binding of NAD to at least two of the three strong binding sites was accompanied by significant changes in the sedimentation coefficient of the protein (after correcting for the contribution of the bound coenzyme to the buoyant weight). However, no unequivocal interpretation of these results could be made for several reasons. The preparations of apoGPDH to which various amounts of NAD were added were shown by sedimentation equilibrium studies to contain different molecular weight species (Figure 1). Both dissociated forms of the tetrameric enzyme and slight amounts of aggregate were observed as had been reported previously by Hoagland and Teller (1969). Our results are consistent with their interpretation that the apoenzyme undergoes a slow irreversible conversion to subunits followed by aggregation. The presence of slight amounts of different molecular weight species raised the possibility that the observed changes in sedimentation coefficients accompanying NAD binding might be due to slight shifts in the association–dissociation equilibria. Attempts to determine whether NAD binding displaced such an equilibrium, including experiments at pH 9 where the extent of dissociation of the enzyme was shown to be greater (Figures 2 and 3), proved not fruitful largely because of the lability of the apoenzyme. After removal of NAD the apoenzyme slowly denatures as indicated by the progressive loss of activity (Figure 4) and the formation of small amounts of dissociated and aggregated species. Because of this denaturation of the protein there was a lack of reproducibility in differential sedimentation experiments on the enzyme in aqueous buffers. Accordingly no results were presented. It was concluded, therefore, that the observed changes in sedimentation coefficient might be due to changes in the frictional factor of the tetrameric enzyme, to shifts in the association–dissociation equilibria, to an alteration by NAD of the rate of denaturation of the enzyme, or to a combination of these effects. Therefore no definitive interpretation was possible. It should be noted, however, that the largest change in sedimentation coefficient occurred upon binding the first two NAD molecules and that binding at the first two sites resulted in almost total prevention of the rapid precipitation observed with high concentrations of aqueous solutions of apoGPDH.

Since the results obtained in the aqueous buffer could not be interpreted in terms of conformational changes, an environment was sought in which the enzyme was stabilized in the tetrameric form. It was found that both the enzyme and the apoenzyme in D_2O buffers were not inactivated or precipitated (Figure 4); moreover this medium maintained the enzyme as tetramers at room temperature. D_2O has been reported to affect the quaternary structure of a number of other proteins, in some cases resulting in greater stabilization. For example, it has been found that both glutamate dehydrogenase (Henderson and Henderson, 1969) and lactate dehydrogenase (Henderson *et al.*, 1970) are stabilized by D_2O against irreversible denaturation and against dissociation. Similarly D_2O enhances the dimerization of α-chymotrypsin (Aune *et al.*, 1971) and facilitates aggregation of tobacco mosaic virus protein (Khalil and Lauffer, 1967), β-galactosidase (Erickson and Steers, 1970), spindle microtubular proteins (Inoué and Sato, 1967), and β-lactoglobulin (Baghurst *et al.*, 1971). D_2O also increases the stability of RNA and ribosomes (Lewin and Williams, 1971) and apparently has general effects on a number of biological systems (Kritchevsky, 1960).

There has been considerable theoretical and experimental work directed toward elucidating the effect of D_2O in stabilizing proteins by strengthening noncovalent interactions such as hydrogen and hydrophobic bonds (Nemethy and Scheraga, 1964; Kresheck *et al.*, 1965; Appel and Yang, 1965; Emerson and Holtzer, 1967; Berns and Lee, 1968; Henderson *et al.*, 1970). However, in view of our limited knowledge about the noncovalent interactions in GPDH it seems inappropriate to apply the considerations about the role of D_2O to the experimental results presented here for GPDH. It is worth noting that the solutions of E, EN, and EN_4 in D_2O, which showed no evident dissociation at room temperature, did exhibit slight dissociation in the same concentration range (0.1–3 g/l.) when the temperature was lowered to 4°. Before these results are interpreted in terms of hydrophobic bonds further work is necessary under different conditions of pH, ionic strength, temperature, and protein concentration.

Although D_2O exerted a strong effect on the structure of the enzyme it did not alter significantly the NAD binding characteristics. The coenzyme is bound at the first two sites with very high affinity, at the third site with a somewhat lower affinity and at the fourth site still more weakly (Figure 6). The enzyme, therefore, exhibits negative cooperativity in D_2O solutions. In view of the report of Velick *et al.* (1971) that the binding of NAD exhibited a strong temperature dependence, it would be of considerable interest to repeat the binding experiments in D_2O at different temperatures. It is possible that temperature has a greater and perhaps different effect on the structure of the enzyme in H_2O than it does when the enzyme is in D_2O.

Sedimentation equilibrium studies showed that the tetrameric form of the enzyme, both in the absence and presence of NAD, was stabilized markedly in D_2O solutions. This conclusion was reinforced by the differential sedimentation experiments which showed that the concentration dependence of the sedimentation coefficient was independent of the amount of bound NAD and that the values of k_{app} (Table II) were characteristic of noninteracting globular proteins (Smith *et al.*, 1973). Additional support for this conclusion came from several experiments (Table III) which showed that $\Delta s/s_{av}$ caused by NAD binding did not vary significantly with protein concentration. Hence we can conclude that the observed changes in sedimentation coefficient upon NAD binding in D_2O solutions were attributable to the increase in mass of the sedimenting unit due to the ligand and/or changes in the shape or volume of the protein molecules rather than to shifts in the association–dissociation equilibrium involving tetramers and dimers, for example.

As seen in Figure 8, there was a significant increase (amounting to 4%) in the measured sedimentation coefficient of the protein upon the binding of four NAD molecules to each tetramer. It is tempting to interpret this change in terms of a

decrease in the frictional coefficient of the protein which, in turn, could be attributed to shrinkage and/or a change in the shape of the sedimenting molecules to a more symmetric form. This type of reasoning was invoked to account for the effect of ligands on the conformation of aspartate transcarbamylase (Gerhart and Schachman, 1968). However, interpreting the experimental results on GPDH is much more difficult since the bound ligands contribute significantly to the molecular weight (1.8%) and their density is much greater than that of the protein. Hence their contribution to the buoyant weight of GPDH, calculated according to eq 1, is greater than 3%. It should be recalled that the binding of ligands to aspartate transcarbamylase was accompanied by a *decrease* in the sedimentation coefficient despite the slight increase in the buoyant weight of the sedimenting unit (Gerhart and Schachman, 1968). Such a result can be interpreted unambiguously as resulting from an increase in the frictional coefficient (Smith *et al.*, 1973). With GPDH the situation is more complicated since the measured increase in the sedimentation coefficient is only slightly larger than that which can be attributed to the contribution of the bound NAD to the buoyant weight. Figure 8 shows that the corrected values for $\Delta s/\bar{s}_{av}$ for two different enzyme preparations are very small.[3]

In making the corrections for the bound NAD we assumed that the volumes of the protein and the ligand were additive and we used our measured value, 0.56 ml/g, for the specific volume of NAD in D_2O. This assumption is critical, as is the value for the specific volume of the ligand. There is as yet no information regarding the partial specific volumes of complexes such as EN and EN_2. When such data become available and uncertain electrostriction effects can be evaluated, we would be in a better position to assess the validity of the corrected values of $\Delta s/\bar{s}_{av}$ shown in Figure 8. It is possible that the corrections may not be exactly proportional to the number of NAD molecules bound to the protein. Further progress in elucidating the detailed structure of GPDH (and the location of the NAD molecules) from X-ray diffraction studies (Watson *et al.*, 1972) should provide useful information on this possibility. In the meantime we have no recourse other than to assume additivities of volumes in making the required corrections.

As indicated in Figure 8 for one of the enzyme preparations, the binding of the first NAD molecule to the tetramer causes a hydrodynamic change which is different (negative) from those produced by the binding of subsequent NAD molecules. This initial change, reflected by the decrease in sedimentation coefficient, may be a gross, concerted conformational transition involving the swelling of the molecules due to a change in the packing and orientation of the subunits within the tetramers. There may be, in addition, a restricted local change in the tertiary structure leading to a tightening in the folding of the particular polypeptide chain to which the NAD molecule is bound. Subsequent local changes in the other chains upon binding of NAD to them could lead progressively to a more compact structure with an increased sedimentation coefficient. Although it is not possible at this stage to study the effect of NAD on the isolated subunits, there is a precedent for ligands producing hydrodynamic changes in isolated subunits which are opposite to the changes produced in the intact oligomers. The binding of succinate and carbamyl phosphate to the isolated catalytic subunit of aspartate transcarbamylase causes an increase of about 1% in the sedimentation coefficient (Kirschner and Schachman, 1971b) whereas the binding of the same ligands to the intact enzyme causes a decrease of 3.6% (Gerhart and Schachman, 1968).

The data for the second preparation of GPDH shown in Figure 8 vary somewhat from those discussed above. We have no explanation for the slight, systematic differences in sedimentation behavior among the various preparations. One additional sample, even in D_2O, showed a tendency to aggregate upon the removal of all the bound NAD by the treatment with charcoal. Perhaps this difficulty could be remedied by preparing the apoenzyme in D_2O solutions. It should be noted that the differences in the behavior of the two samples (for which data are presented in Figure 8) are extremely small. No break in the curve is evident for the sample depicted by the squares; however the scatter in the data for this preparation was significantly greater than for the other sample (see the circular data points in Figure 8). Resolving this apparent slight discrepancy will require more reproducible preparations and even greater precision in the sedimentation measurements.[4]

[3] An additional correction could be made, in principle, for the NAD contribution to the friction factor of the sedimenting complex. The exact magnitude of this correction depends on the degree to which the NAD molecules are buried within the protein. As a first approximation it can be assumed that each bound NAD molecule increases the molar volume of the complex by the value of the molar volume of the NAD molecule itself (Schumaker, 1968). Since the friction factor is proportional to the $^1/_3$ power of the volume, such a correction would be very small. Until there is further knowledge about the three-dimensional structure of GPDH and the location of the four NAD molecules it seems premature to make any correction of this type. Hence, the corrected values of $\Delta\bar{s}/\bar{s}_{av}$ in Figure 8 can be used tentatively to give an estimate of the change in the frictional coefficient of the protein upon the binding of four NAD molecules. For symmetric molecules the results indicate an increase of 0.3–0.5% which corresponds to a volume decrease only slightly greater than 1%.

[4] While this paper was in press, the results of similar studies on the effect of NAD on the sedimentation of GPDH in D_2O were reported by Noel and Schumaker (1972). Although the magnitude of the observed change in the sedimentation coefficient upon the binding of four NAD molecules to the tetramers was the same in the two studies, there are some marked discrepancies which merit comment. They performed fewer experiments at low ratios of NAD to protein; hence if the binding of the first NAD molecule caused a different effect from that of subsequent NAD molecules, the nonlinear dependence of $\Delta\bar{s}/\bar{s}_{av}$ on r would not have been detected. Since our experiments showed slightly different results with various preparations it is obvious that further studies are required to clarify this issue. There is substantial disagreement between us in evaluating the magnitude of the correction for the contribution of the bound NAD to the buoyant weight of the sedimenting unit. They used an estimated partial volume of 0.62 ml/g for NAD whereas our correction was based on the measured value of the specific volume. In addition we used the density of D_2O solutions in calculating the correction factor. In our view their estimate of the change in the frictional coefficient of the protein upon NAD binding was too high. Noel and Schumaker (1972) found a much larger concentration dependence for the sedimentation coefficient of both the apoenzyme and the holoenzyme than are shown in Table II. Their values are not consistent with the results usually found for globular proteins and are in marked disagreement with the values for GPDH reported by Fox and Dandliker (1956a) and by Harrington and Karr (1965). Similarly their values of the sedimentation coefficient at infinite dilution are much higher than our results and others in the literature, but we have been informed (V. Schumaker, private communication) that their reported values are too high because of their use of an incorrect magnification factor for the optical system of the ultracentrifuge. We differ also in that our sedimentation equilibrium experiments on apoGPDH in D_2O solutions showed very little dissociation of the tetramers whereas they found a marked dissociation as revealed by the decrease of the sedimentation coefficient in dilute solutions. It is possible that the use of D_2O for their preparations was not completely effective in stabilizing the tetrameric form of the enzyme. If so, then the observed changes in sedimentation coefficient upon NAD binding cannot be interpreted unambiguously in terms of alterations in the frictional coefficient.

The alteration of the sedimentation coefficient of GPDH upon NAD binding is consistent with a large body of evidence that NAD exerts a structural role in the molecule (Velick and Furfine, 1963; Colowick *et al.*, 1966; Zavodszky *et al.*, 1966; Conway and Koshland, 1968; Constantinides and Deal, 1969; De Vijlder *et al.*, 1969; Hoagland and Teller, 1969; Fenselau, 1970b). Some studies indicate that the effect of the first NAD on the conformation of the enzyme is qualitatively different from the effects of binding at the remaining sites. First, Fenselau (1970b) has shown that the binding of the first NAD leads to over 50% of the total stabilization of the molecule against proteolytic enzymes. Second, De Vijlder and Slater (1968) showed that the first NAD binds more rapidly than the second, third, and fourth molecules. Third, Conway and Koshland (1968) showed that binding of the first NAD causes a maximal increase in the reactivity of the active-site SH groups. Fourth, Listowsky *et al.* (1965) showed that the major part of the change in the optical rotatory dispersion of the enzyme occurs after the first molecule has bound. Although alternative explanations have been proposed for the latter two observations, evidence from a variety of studies strongly suggests that the first NAD to bind is unique in its conformational effect on the molecule. Most recently Simon (1972) showed that the binding of the first NAD molecule to GPDH caused a greater decrease in the radius of gyration of the protein than did the binding of subsequent molecules.

In view of the marked stabilization of the tetrameric form of the protein by D_2O, it would be useful to reexamine these effects in that medium. Such experiments should indicate the extent to which the various properties are influenced by changes in the association–dissociation equilibria. In this way the relative roles of conformational changes in the tertiary and quaternary structures may be assessed in terms of the negative cooperativity of binding NAD. Although the present studies demonstrated that changes in the tetrameric form of the protein could be studied in D_2O without the complication of dissociation into subunits, it should be recognized that association–dissociation reactions may be important for the enzyme's function *in vivo*. At the concentrations and local environment at which the enzyme exists in the cell, NAD may well have a function in influencing the association–dissociation equilibria between the tetramer and its subunits.

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Analysis of Association–Dissociation Equilibria in Proteins by Difference and Differential Sedimentation†

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ABSTRACT: Although the weight average sedimentation coefficient, s, of an oligomeric protein is affected frequently by the addition of specific ligands, it is not clear generally whether this effect is due to a shift in the association–dissociation equilibrium of the protein or to a conformational change leading to an altered frictional coefficient. This ambiguity can be eliminated, in part, by combining measurements of the change in sedimentation coefficient, Δs, at one protein concentration with data for the effect of the ligand on the concentration dependence of the sedimentation coefficient, ds/dc. Equations are presented relating Δs at a single protein concentration to a ligand-promoted shift in the association constant for a monomer–dimer system. Analogous equations are derived relating the association constant (and changes in it) to the observed values of ds/dc. Two types of self-associating systems are considered: in one the extent of association is very high with the protein being mainly in the dimeric form; for the other the protein is largely in the dissociated form as monomer. The theoretical treatments show how these two types of system differ with regard to the values of Δs and the changes in ds/dc when ligands perturb a monomer–dimer equilibrium. Extremely small changes in Δs can be measured accurately and directly by the recently developed difference sedimentation technique based on interference optics. Accurate measurements of ds/dc are readily obtained by the differential sedimentation technique in which differing concentrations of the same protein solution are layered over one another in a synthetic boundary cell. The assumptions and limitations inherent in the use of these sedimentation velocity techniques for detecting and measuring shifts in the self-association of proteins are discussed in relation to the theoretical treatment.

Many proteins are known to undergo conformational changes in response to the binding of substrates and ligands, and much work is being devoted to the determination of the nature and magnitude of these changes. Such effects are manifested either as alterations in the secondary and tertiary structures of the individual chains themselves or in the state of aggregation of these chains within oligomers, or to a combination of both factors. Among the many physical-chemical techniques used for examining such changes, hydrodynamic methods such as sedimentation velocity have been found to be particularly fruitful since the measured parameter, the sedimentation coefficient, is sensitive to both the molecular weight and the shape of the kinetic unit (Svedberg and Pedersen, 1940).

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