Influence of Substrates on the Dissociation of Rabbit Muscle D-Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: A preparation of rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase which dissociates reversibly at neutral pH has been studied. The system can be described by a dimer–tetramer reversible equilibrium with association constant of 2×10^6 l./mole at pH 7 and low ionic strength at 5°. When added together, diphosphopyridine nucleotide and phosphate shift the equilibrium toward tetramer ($k_2 = 5 \times 10^6$ l./mole). Either substrate alone does not produce an effect. Acylation of the enzyme by the combination of glyceraldehyde, phosphate, and diphosphopyridine nucleotide produces an irreversible dissociation to dimer. It is concluded that the dissociated species is the ϵ-aminoacyl enzyme of Mathew *et al.* (Mathew, E., Meriwether, B. P., and Park, J. H. (1967), *J. Biol. Chem.* 242, 5024). *p*-Nitrophenyl acetate is also a specific perturbant of the equilibrium. Active enzyme sedimentation studies (Cohen, R., and Hahn, C. (1965), *Compt. Rend. 260*, 2077) have been performed to determine the size of the catalytic unit. As a dehydrogenase, only the tetrameric form of the enzyme is active. Esterase activity is present in the dimer and apparently also in the tetrameric state of association. Studies with the apoenzyme both by short-column sedimentation equilibrium and by sedimentation velocity indicates that the preparation initially consists of both dimer and tetramer. After longer periods, the enzyme further dissociates to monomer as well as aggregates to a high degree. Finally, a computation procedure has been developed for the determination of equilibrium constants for highly associated systems of dimers and tetramers (or monomers and dimers). In addition a method of accurately calculating the molecular weight of the smallest component in such systems is proposed and utilized.

The molecular weight of mammalian muscle D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) has been investigated in several laboratories. Molecular weight determinations by sedimentation velocity and diffusion yielded values of approximately 120×10^3 (Taylor *et al.*, 1956; Elias *et al.*, 1960) and 143×10^3 g/mole (Elodi, 1958). Dandliker and Fox (1955) and Fox and Dandliker (1956a,b) found 140×10^3 by light scattering and 137×10^3 g/mole by sedimentation velocity and diffusion. Using the Archibald method, Elias *et al.* (1960) found a molecular weight of 118×10^3. More recently Harrington and Karr (1965) have observed 145×10^3 g/mole using both sedimentation velocity and sedimentation equilibrium. Jaenicke *et al.* (1968) have found the same value for the native enzyme and have also concluded that the tetramer does not dissociate in solution except at extremes of pH, ionic strength, and in the presence of detergents.

Harris and Perham (1965) have reported that glyceraldehyde 3-phosphate dehydrogenase is composed of four identical subunits. Harrington and Karr (1965) have shown that the enzyme is dissociated to a monomer of 36.3×10^3 g/mole in 5 M guanidine hydrochloride. The dissociation reported by Jaenicke *et al.* (1968) was also found to proceed to the monomer stage. Elodi (1958) claimed that KCN produced a halving of the molecular weight but Elias *et al.* (1960) did not observe this effect, nor have we been able to repeat it.

We have found a dimer–tetramer chemical equilibrium at pH 7 in low ionic strength at 5°. This equilibrium is affected in various ways by substrates of the enzyme. Since the physical state of the catalytic unit had not been determined either for dehydrogenase or esterase activities, we have examined this problem. These results correlate rather well with thermodynamic observations on the dimer–tetramer equilibrium. Finally, we have studied the physical state of the apoenzyme and from the results are able to propose a hypothesis for the stages of denaturation of this species with time.

Theory

In order to calculate point-by-point equilibrium constants the number-, weight-, and average molecular weights at each point (denoted by $M_{n,r}$, $M_{w,r}$, and $M_{z,r}$, respectively) were utilized. The point-by-point equilibrium constants are given by the relations

$$\frac{2k_{2,n}}{M_1} = \frac{2M_{n,r}(M_{n,r} - M_1)}{C(2M_1 - M_{n,r})^2} \quad (1)$$

$$\frac{2k_{2,w}}{M_1} = \frac{M_1(M_{w,r} - M_1)}{C(2M_1 - M_{w,r})^2} \quad (2)$$

$$\frac{2k_{2,z}}{M_1} = \frac{(M_{z,r} - M_1)(3M_1 - M_{z,r})}{4C(2M_1 - M_{z,r})^2} \quad (3)$$

where M_1 is the molecular weight of the smallest species

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which participates in the equilibrium (a dimer in the case of D-glyceraldehyde 3-phosphate dehydrogenase). C is the concentration in grams per liter. The subscript in the $k_{2,i}$ simply indicates that the constant was derived from the ith molecular weight moment; all $k_{2,i}$'s are molar association constants. Equation 1 was derived by Nichol *et al.* (1964), while eq 2 was given by Rao and Kegeles (1958).

In order to compute the best point-average constant, the following weighted average was used

$$\bar{k}_2 = \Sigma_i w_i k_{2,i} / \Sigma_i w_i \tag{4}$$

where the summation in i is over the number-, weight-, and z-average constants. The weights are the sums of the reciprocal absolute deviations of observed and predicted molecular weights; for example

$$w_n = |M_{w,r} - \tilde{M}_{w,r}|^{-1} + |M_{z,r} - \tilde{M}_{z,r}|^{-1}$$

where the tilde above the symbol indicates the value of the molecular weight predicted from C, $k_{2,n}$, and M_1. The estimate of the reliability of each $\bar{k}_2$ which was used is

$$E(\bar{k}_2) = \left[\frac{\Sigma_i w_i k_{2,i}^2}{\Sigma_i w_i} - \bar{k}_2^2\right]^{1/2} \tag{5}$$

This function is the weighted root-mean-square uncertainty of the constant derived at each point in the cell. Values of $\bar{k}_2$ and $E(\bar{k}_2)$ were computed at concentrations greater than 0.25 g/l.

In order to characterize the reaction by a single equilibrium constant, we constructed another weighted average

$$\hat{k}_2 = \Sigma_j f_j \bar{k}_{2,j} / \Sigma_j f_j \tag{6}$$

where j is summed over all data points greater than 0.25 g/l. The weighting factors, f_j, are the values of $1/E(\bar{k}_{2,j})$ determined at each point by eq 5.

The root-mean-square uncertainty in $\hat{k}_2$ is the same as in eq 5

$$E(\hat{k}_2) = \left[\frac{\Sigma_j f_j \bar{k}_{2,j}^2}{\Sigma_j f_j} - \hat{k}_2^2\right]^{1/2} \tag{7}$$

This expression appears to give an overestimate of the error since back-calculated distributions for weight-average molecular weights often show that more than 90% of the points lie below the line predicted by $\hat{k}_2 + E(\hat{k}_2)$ while all points lie above the line predicted by $\hat{k}_2 - E(\hat{k}_2)$. In this report, uncertainties in equilibrium constants will not be stated unless the error bars in M_n and M_w include about 60% of the data. It seems pointless to give an uncertainty figure which encompasses all of the data and occasionally leads to negative values of $\hat{k}_2 - E(\hat{k}_2)$ for highly associated systems.

Since real systems have activity coefficients which affect the molecular weight moments, we also calculated expressions 1–4 as linear functions of C. The virial terms enter into the $k_{2,i}$ (apparent) in complex ways but were not required for description of the data presented here.

Materials and Methods

DL-Glyceraldehyde 3-phosphate diethyl acetal barium salt, D-glyceraldehyde, sodium pyruvate, sodium lactate, DPN, and DPNH were purchased from Sigma Chemical Co. Glyceraldehyde 3-phosphate was prepared by deionizing on Dowex 50 and heating in boiling water for 3–5 min. The concentration of D-glyceraldehyde 3-phosphate was determined enzymatically with DPN and glyceraldehyde 3-phosphate dehydrogenase. The total concentration of the D and L forms was measured by release of P_i upon base hydrolysis.

These solutions were frozen and used within 3 days after thawing. In spite of these precautions, the sedimentation equilibrium experiments gave variable results presumably due to alkaline decomposition of the glyceraldehyde 3-phosphate (Sigma Technical Bulletin, 1961). Glyceraldehyde was also purchased from Sigma Chemical Co. Weighed samples of the polymer were dissolved in boiling water and always used within 4 hr.

Crystalline rabbit muscle glyceraldehyde 3-phosphate dehydrogenase was prepared and generously donated by Dr. Marian Kochman of this Department (Kochman and Rutter, 1968). The sedimented crystals were dissolved in buffer to a concentration of about 30 g/l. and then dialyzed overnight against the same buffer at 4°. The pH of all buffers was adjusted and measured at room temperature. Apoglyceraldehyde 3-phosphate dehydrogenase was best prepared by passing the dialyzed enzyme solution over a 0.4 × 20 cm Norit–cellulose column (Fox and Dandliker, 1956a). Alternatively, the enzyme solution was stirred for 20 min with 100 mg of Norit and then centrifuged. This procedure was repeated until the ratio of the absorbance at 280 mμ to that at 260 mμ reached a maximum value (1.85–1.90). Protein concentration was measured spectrophotometrically using the absorbancy index at 280 mμ of 1.00 cm^2/mg for the holoenzyme (Velick *et al.*, 1953) and 0.829 cm^2/mg for the apoenzyme (Fox and Dandliker, 1956a). Enzyme activity assays were conducted in a manner similar to that of Velick (1955) on a Zeiss PM-QII or a Gilford 2000 spectrophotometer. The assay consisted of adding 0.10 ml of a 0.15 M sodium arsenate solution, 0.05 ml of a 0.015 M solution of DPN, and 0.05 ml of a 0.015 M solution of glyceraldehyde 3-phosphate to 2.75 ml of a sodium pyrophosphate buffer (pH 8.5). The reaction was initiated by adding 1 μg of enzyme in 0.05 ml of buffer. The increase in absorbance was followed at 340 mμ. Measurements of absorbance were made at 15 and 45 sec. Twice the difference between these two readings was used as a standard measure of activity. The specific activity of the enzyme was 55 IU/mg of enzyme.

The partial specific volume used in calculations at 20° was 0.739 and 0.729 ml per g at 5° (Taylor *et al.*, 1956). Solvent densities were measured pycnometrically at 20.0°.

Ultracentrifugation was conducted in a Spinco Model

E analytical ultracentrifuge equipped with an electronic speed control. The optical system was focused at the two-thirds plane of the cell (Svensson, 1954, 1956; Yphantis, 1964). The molecular weight determinations were made using the high-speed sedimentation equilibrium technique of Yphantis (1964) with lower speeds and higher initial protein concentrations. The time to reach sedimentation equilibrium was reduced by using an overspeeding–underspeeding technique (Teller *et al.*, 1968).

Centrifuge runs with an initial protein concentration of 0.25 to 0.75 g l. and column heights of 3 mm were generally carried out at 20,000 rpm for 4 hr after which the speed was reduced to 15,000 rpm for 12–15 hr; the six-channel centerpiece described by Yphantis (1964) was used. Experiments utilizing shorter column heights and lower initial protein concentrations were performed at 22,000–32,000 rpm in an eight-channel cell described by Yphantis (1960). All sedimentation equilibrium experiments were done at 5° with a false cell base (Ginsburg *et al.*, 1956) of FC.43 (3 M Co.).

The fringe patterns on the photographic plates from sedimentation equilibrium experiments and water–water base-line runs were read on a modified Nikon microcomparator (Teller, 1967). Points were read through the cell until the fringes could no longer be resolved at the base of the cell. Data were processed with computer programs developed in this laboratory.

The sedimentation velocity experiments were carried out in double-sector cells at 5.5 or 20°. Many of the concentration-dependent experiments were conducted simultaneously using a Spinco AN-F four-hole rotor at 52,000 rpm and cells with wedge windows.

Band sedimentation velocity experiments on the active enzyme were performed using the technique of Cohen and Hahn (1965). Double-sector centerpieces were constructed according to the specifications of Vinograd *et al.* (1963). The layering reservoir contained 25 μl of the enzyme. The sectors contained the usual activity assay solution with the addition of 0.1 M NaCl to stabilize the layering of the band. The movement of the band was followed by the increase in absorption at 340 mμ using a Spinco absorption scanning system. The band sedimentation velocity experiments with the apoenzyme and *p*-nitrophenyl acetate were conducted at 20° using 15 μM *p*-nitrophenyl acetate and 5% sucrose in 0.1 M Tris–1.0 mM EDTA (pH 7.5). Movement of the band was followed at 400 mμ.

Results

Glyceraldehyde 3-phosphate dehydrogenase at sedimentation equilibrium in the analytical ultracentrifuge exhibits molecular weight distributions such as shown in Figure 1. Figure 1A depicts the weight-average molecular weight data as a function of concentration while Figure 1B shows the number-average data. Because the molecular weights decrease at low concentration, the tetrameric molecule is not the only species present in these solutions. Owing to uncertainties in fringe labeling methods (Yphantis, 1964; Teller *et al.*, 1968), it is difficult to be sure whether such dissociation is characteristic of an artifact of computer techniques, contamination of the preparation by small material, or a true chemical equilibrium.

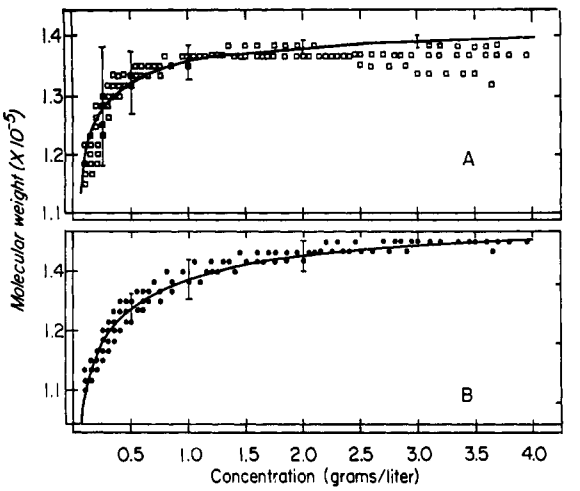

FIGURE 1: Weight- and number-average molecular weights. (A) Weight-average molecular weights as a function of concentration. (B) Number-average molecular weights as a function of concentration. The solid lines are those drawn for $k_2 = 2.3 \times 10^6$ l./mole. The error bars represent the potential error in molecular weight due to uncertainty in fringe labeling (Yphantis, 1964; Teller *et al.*, 1968). These bars are averages of the three channels of data and are drawn centered on the molecular weights predicted by k_2. These data are from line 5 of Table I.

Regardless of whether the dissociation was a true equilibrium, we wished to further characterize its nature in order to perform other experiments with this enzyme. Figure 2 shows the data from $M_{w,r}$ *vs.* $1/M_{n,r}$, $M_{z,r}$ *vs.* $1/M_{w,r}$, and $(2M_{w,r} - M_{z,r})$ *vs.* $(2/M_{n,r} - 1/M_{w,r})$ (Yphantis and Roark, 1968; Teller *et al.*, 1968) from the experiment of Figure 1. Because of large experimental error at low concentration these data do not unambiguously prove that the dissociation was the tetramer–dimer type, but are indicative of this mechanism of dissociation. Consequently we performed the following calculation. A dimer–tetramer chemical equilibrium was assumed and the molecular weight of the dimer was varied from 68×10^3 to 81×10^3 g per mole by increments of 1×10^3 g/mole. From each value of $M_{n,r}$, $M_{w,r}$, and $M_{z,r}$ values of $k_{2,i}$ were calculated and averaged as described in the Theory section. From $\hat{k}_2$, C, and M_1 (assumed) the average deviation of the $M_{n,r}$ and $M_{w,r}$ data was calculated both for all data and the data used in deriving $\hat{k}_2$. These average deviations were found to be nearly parabolic functions of M_1 (assumed). The minimum of the curves was taken as the true value of the molecular weight of the dimer. This type of "search" routine was performed on four data sets of Table I with the averaged result of $72 \pm 1 \times 10^3$ g/mole for the molecular weight of the dimer.

These computations together with graphs similar to that of Figure 2 for other experiments firmly established that the system could be described by a tetramer–dimer dissociation but could not be used to determine whether a true chemical equilibrium was occurring. Essentially the same molecular weight distributions would be observed for an irreversible dissociation of the tetramer.

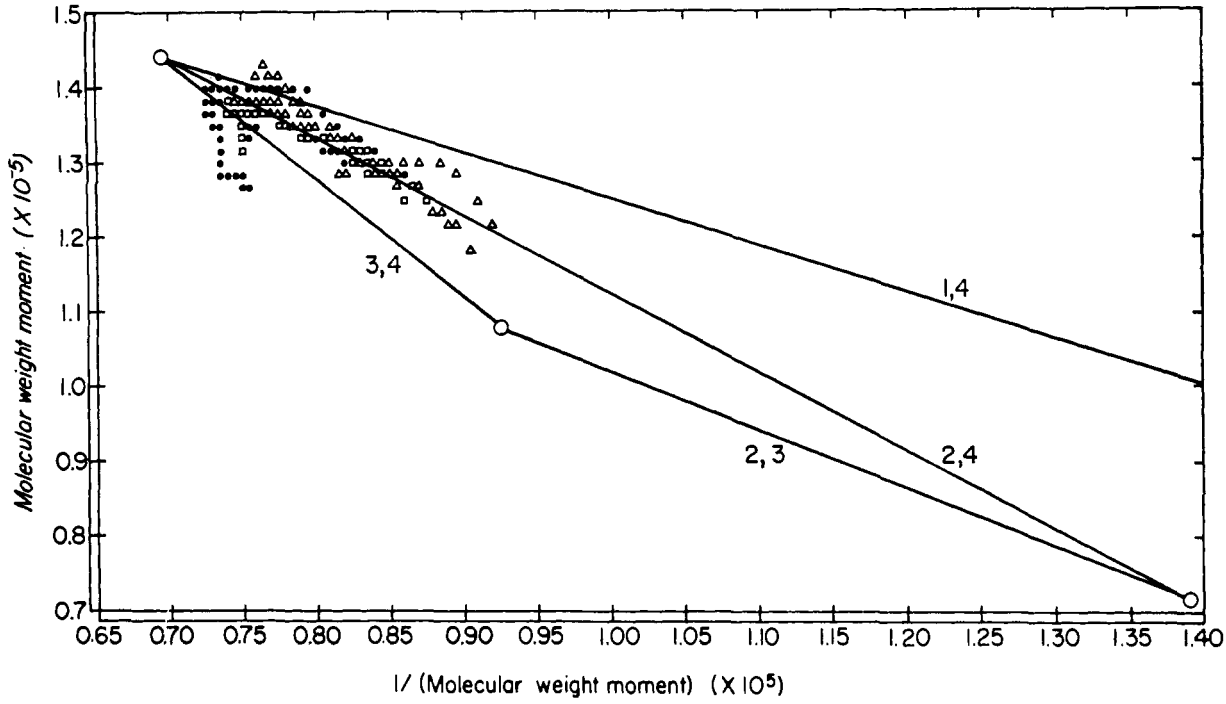

FIGURE 2: Sedimentation equilibrium data from Figure 1. Filled circles are $M_{z,r}$ *vs.* $1/M_{w,r}$ data. Open squares are $M_{w,r}$ *vs.* $1/M_{n,r}$ data. Open triangles are $2M_{w,r} - M_{z,r}$ *vs.* $2/M_{n,r} - 1/M_{w,r}$ data. The downward trend of the filled circles and open squares is due to thermodynamic nonideality (Yphantis and Roark, 1968; Teller *et al.*, 1968).

In order to establish that a solute system is in chemical equilibrium it is necessary to establish that point-by-point molecular weight moments are functions only of concentration (Adams and Fujita, 1963). Figure 3 shows a high-speed sedimentation equilibrium experiment with D-glyceraldehyde 3-phosphate dehydrogenase at initial concentrations of 0.25, 0.50, and 0.75 g per l. Figure 3A depicts the number-average molecular weights and demonstrates that they are reasonably superimposible. $M_{w,r}$ and $M_{z,r}$ data (Figure 3B, C, respectively) deviate in the three channels in a manner expected for a system with a dimer–tetramer equilibrium contaminated by material of molecular weight greater than that of the tetramer. Graphs such as Figure 2 have been used to indicate that this material is probably octamer. This large material introduced a complication which we have not been able to control. Further, its presence tends to increase apparent equilibrium constants. The value of k_2 determined from this experiment was $1.7 \pm 0.9 \times 10^6$ l./mole (Table I, line 1). Using this constant the M_n and M_w data are fit rather well suggesting that the heavy material actually composes only a few per cent of the original preparation.

The next criterion of a chemical equilibrium which we applied was that experiments in 30-mm centerpieces should yield the same association constant as those performed with 12-mm centerpieces. This is the case as demonstrated by the third line of Table I. In the absence of effectors which perturb the equilibrium the system can be described by an association constant of $2.0 \pm 0.5 \times 10^6$ l./mole.

The third test of chemical equilibrium, that of passing the material through Sephadex G-200 and centrifuging the fraction of highest specific activity, led to the ambiguous result that a large amount of octamer was observed

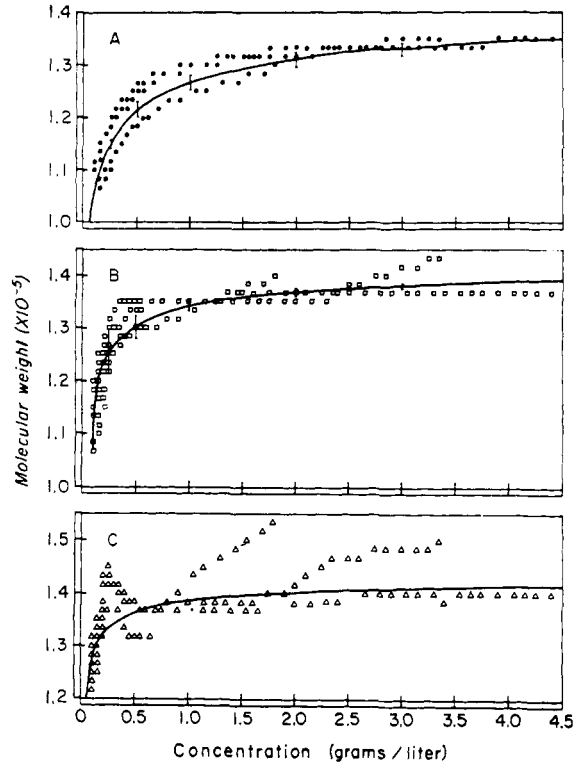

FIGURE 3: Sedimentation equilibrium data with D-glyceraldehyde 3-phosphate dehydrogenase at three different initial concentrations. (A) Number-average molecular weights. (B) Weight-average molecular weights. (C) *z*-average molecular weights. The lines are drawn for $k_2 = 1.7 \times 10^6$ l./mole. The error bars represent the potential errors in molecular weight due to uncertainty in fringe labeling (Yphantis, 1964 Teller *et al.*, 1968). These bars are averages of the three channels and are centered on the molecular weights predicted by k_2. These data are from line 1 of Table I.

(about 10%). Nevertheless, the initial $M_{n,r}$ and $M_{w,r}$ *vs.* C data follow the molecular weight distributions of the experiments presented in rows 1–3 of Table I.

It is difficult to establish exactly a dissociation reaction mechanism in a system contaminated with components not participating in the chemical equilibrium. However, in this case, the evidence for equilibrium between the dimer and tetramer is rather compelling since we are able to perturb the equilibrium by various specific combinations of effectors. The data presented in Table I summarize some of the experiments which have been performed in attempts to perturb the chemical equilibrium.

In rows 4–7 it may be seen that inorganic phosphate alone or together with lactate, pyruvate, or DPNH has no effect on the dimer–tetramer distribution. Row 8 indicates that DPN in Tris buffer yields similar results. However, the combination of DPN and phosphate raises the association constant by a factor of 2–3 as may be observed in the experiments summarized by rows 9 and 10 of this table. Another specific perturbation of the equilibrium is demonstrated by the next three rows of this table (11–13). Glyceraldehyde together with phosphate has no effect upon the equilibrium but the combination of glyceraldehyde, phosphate, and DPN results in a marked dissociation of the tetrameric enzyme. Rows 12 and 13 show that $\hat{k}_2$ is decreased by a factor of 10.

Enzymatic dehydrogenase activity, as determined by initial velocities, is unchanged for all of the ligands except the glyceraldehyde–phosphate–DPN combination. In this case enzyme activity decays as a linear function of time resulting in a halving of activity in 20 hr at 5°. Dialysis of these solutions against 0.1 M Tris-HCl, 10^{-3} M EDTA, and 10^{-3} M 2-mercaptoethanol at pH 7 does not restore the activity although it does eliminate the time-dependent loss of activity. In addition, the 280 mμ/260 mμ ratio drops drastically from its initial value of 1.15 to 0.57, while the control enzyme solution only changed to 1.09.

This result suggests an irreversible modification of D-glyceraldehyde 3-phosphate dehydrogenase by these reagents. Reference to Table I shows that the fit of the M_n and M_w data to the theoretical dimer–tetramer equilibrium is not nearly as good for these experiments as those for the other conditions in the table. This poor fit to the data is not due to the lack of a dimer–tetramer system, but is caused by the lack of superposition of the $M_{n,r}$ and $M_{w,r}$ *vs.* C data between channels. Figure 4 demonstrates that this dissociation to the dimeric state is specific. The observed molecular weight values fall along the line representing a tetramer–dimer dissociation. The presence of monomer or other components would cause divergence of the points from the 2,4 line.

Experiments utilizing glyceraldehyde 3-phosphate in place of glyceraldehyde were quite variable with respect to the apparent association–dissociation properties of the system. Presumably this result was due to the instability of the glyceraldehyde 3-phosphate at neutral and

TABLE I: Equilibrium Constants for D-Glyceraldehyde 3-Phosphate Dehydrogenase with Various Effectors.

Row	Buffer[a]	Effectors (moles/l.)	$k_2 \times 10^{-6}$ (l./mole)	$\pm M_n$[b]	$\pm M_w$[b]	$\pm M_n$[c]	$\pm M_w$[c]
1	Tris	None	1.7 ± 0.9	2726	2644	2132	1852
2	Tris	None	1.54 ± 0.70	2990	2117	1884	1639
3	Tris	None	1.4	3016	5164	2053	2997
4	Phosphate	None	2.8	4759	3791	3455	2592
5	Phosphate	Lactate (0.02)	2.3 ± 0.6	973	1804	872	1503
6	Phosphate	Pyruvate (5×10^{-4})	1.5 ± 0.7	2372	2135	1999	1887
7	Phosphate	DPNH (6.7×10^{-5})	2.5 ± 1.0	2264	2445	1625	1321
8	Tris	DPN (1.5×10^{-3})	1.44 ± 0.73	3893	2668	2570	2842
9	Phosphate	DPN (6×10^{-5})	5.45	4121	2314	2722	1421
10	Tris	DPN (1.5×10^{-3}) Phosphate (0.11)	4.8 ± 0.8[d]	3464[d]			
11	Tris	Glyceraldehyde (0.08) Phosphate (0.11)	1.39	4681	3705	3660	2564
12	Tris	Glyceraldehyde (0.08) Phosphate (0.11) DPN (1.2×10^{-3})	0.206	5274	3856	3684	3764
13	Tris	Same as 12	0.335	4964	3474	3564	3474
14	Tris	10% isopropyl alcohol	2.48 ± 0.25	1127	1684	856	445
15	Tris	10% isopropyl alcohol, *p*-nitrophenyl acetate	$k_2 = 0.20 \pm 0.004$ $k_3 = 0.00089 \pm 0.0036$ $k_4 = 1.84 \pm 0.23$	383	1164		
16	Pyrophosphate	None	0.60 ± 0.04	851	1793	249	1151

[a] Tris = 0.1 M Tris-HCl–0.001 M EDTA–0.001 M 2-mercaptoethanol (pH 7.0); phosphate = 0.1 M sodium phosphate (pH 7.0); pyrophosphate = 0.05 M sodium pyrophosphate (pH 8.5). [b] For all data. [c] For data greater than one fringe. [d] Computed from c *vs.* r^2 data from two individual cells. The error estimate is a composite value of root-mean-square fit to the data.

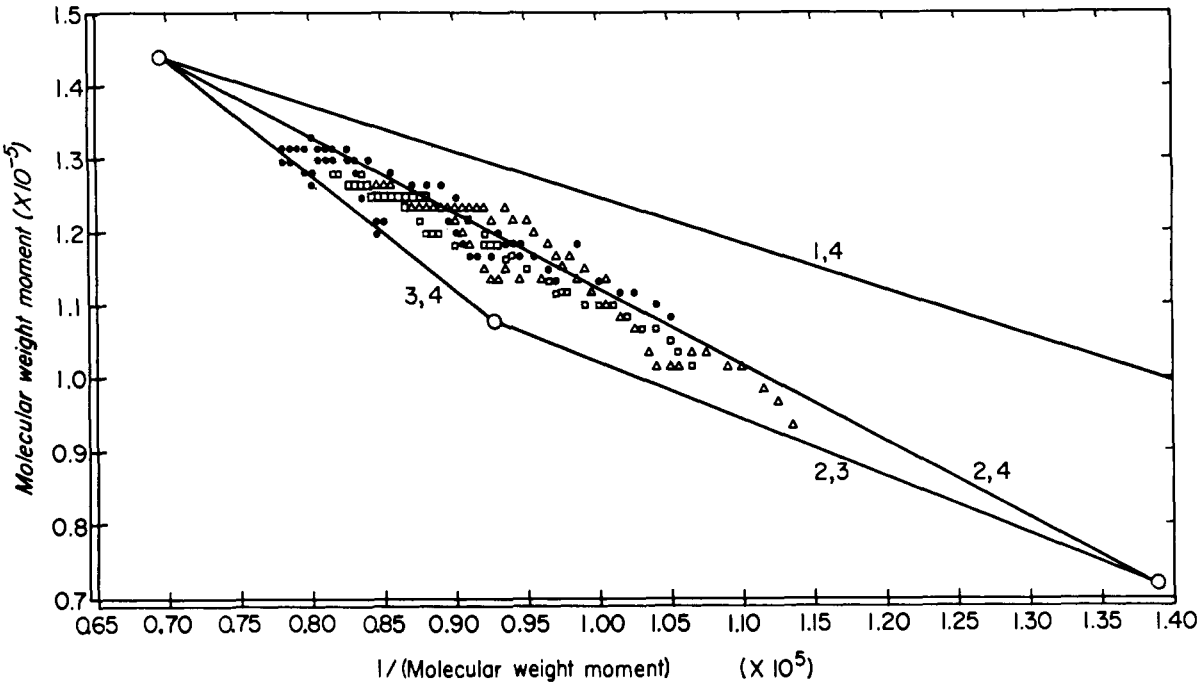

FIGURE 4: Effect of glyceraldehyde, DPN, and phosphate on D-glyceraldehyde 3-phosphate dehydrogenase at pH 7, 5°, in 0.1 M Tris-HCl–10^{-3} M EDTA–10^{-3} M 2-mercaptoethanol. Filled circles are $M_{z,r}$ *vs.* $1/M_{w,r}$; open squares are $M_{w,r}$ *vs.* $1/M_{n,r}$; open triangles are $2M_{w,r} - M_{z,r}$ *vs.* $2/M_{n,r} - 1/M_{w,r}$. Data from two channels are included in the figure.

alkaline pH (Sigma Technical Bulletin, 1961). Occasionally a dissociation comparable with that observed with glyceraldehyde was seen; in other experiments essentially no dissociation was observed.

Row 14 of Table I shows that 10% isopropyl alcohol does not affect the enzyme but the combination of isopropyl alcohol and *p*-nitrophenyl acetate gives both dissociation and higher aggregates as noted in line 15 of the table. The value of k_2 is the same magnitude as observed for glyceraldehyde; k_3 for the reaction $T_2 + T_4 \rightleftharpoons T_6$ is not statistically significant while k_4 for the reaction $T_2 + T_6 \rightleftharpoons T_8$ is quite large. This system consists of dimers, tetramers, and octamers. The system is not an indefinite association since neither $[M_{w,r}(3M_{w,r} - 2M_{z,r})]^{1/2}$ nor $2M_{n,r} - M_{w,r}$ was a constant (D. C. Teller, unpublished data). Contrary to the report by Park *et al.* (1961) we observed equal specific activities for holoenzyme and apoenzyme in esterase assays performed in conjunction with this experiment.

The last row of Table I summarizes an experiment conducted at pH 8.5 and 5° in pyrophosphate buffer. The data show even more dissociation of the tetramer.

Sedimentation equilibrium studies of the apoenzyme were difficult to perform due to instability of this species. Experiments using 2.5-mm solution columns required 16 hr to reach sedimentation equilibrium and were not reproducible. Molecular weight distributions ranged from very low values to very large ones. Occasionally a channel would conform to an indefinite association of dimer but in the same experiment, data from other channels did not verify this model.

In order to characterize molecular weight distributions a short time after formation of the apoenzyme, 1-mm solution columns were used in the eight-channel cell of Yphantis (1960). These solutions reached sedimentation equilibrium in about 4 hr so that the molecular weight distribution should correspond to that present in solutions of apoenzyme which can be fully reactivated. At 4 hr we observed $\hat{k}_2 = 0.41 \times 10^6$ l./mole with a fit to all molecular weight data of ±1117 for $M_{n,r}$ data and ±3275 for $M_{w,r}$ data. From this result it may be concluded that the initial stages of dissociation involve a conversion of tetramer into dimer followed by a later dissociation to monomer together with irreversible aggregation. Addition of DPN to apoenzyme solutions at approximately 4 hr after preparation of the apoenzyme resulted in essentially 100% regain of specific dehydrogenase activity when measured 8 hr after addition of DPN.

Sedimentation velocity studies have also been performed on this system in order to determine whether the dimeric or tetrameric state of the enzyme is the active form. In order to establish the $s^0_{20,w}$ of the enzyme under various conditions, D-glyceraldehyde 3-phosphate dehydrogenase was sedimented at 60,000 rpm using the schlieren optical system and initial concentrations from 2 to 8 g per l. Table II presents the conditions used and the values of the parameters obtained from this experiment.

This table shows that the holoenzyme in the absence of excess DPN has a sedimentation coefficient which is essentially identical with that of the enzyme in the presence of a 40-fold molar excess of DPN. Since the concentrations used for the experiments in this table were greater than 2 g/l. and the amount of dimer is small at this concentration, we regard the sedimentation coefficient of 7.8–7.9 S as that of the tetramer. Apoenzyme, however, gives a decreased $s^0_{20,w}$, corresponding to a

value below that of the tetramer but above that expected for the dimer (4–5 S). Upon aging for 3 days a sample of apoenzyme gave $s_{20,w}$ = 5.3 S which is probably more characteristic of the dimer. The sedimentation coefficient of apoenzyme presented in the table is consistent with the distribution between dimer and tetramer observed in the short-column sedimentation equilibrium experiments. The concentration dependence of the sedimentation coefficients is essentially identical in all cases and quite similar to the usual values for globular proteins (Creeth and Knight, 1965). One difficulty of these experiments is that the addition of DPN approximately 2 hr after its removal does not restore the sedimentation properties to those of holoenzyme (Table II). The sedimentation velocity determinations were made within 2 hr of the addition of DPN. In order to effect complete restoration of enzymatic activity, approximately 8-hr incubation in excess DPN was required. Hence it may be that the structural changes necessary to convert the apodimer to holotetramer are sufficiently slow that the dimer–tetramer distribution of "reconstituted" preparation remained essentially that of the apoenzyme preparation at the time of these experiments.

TABLE II: Sedimentation Velocity Parameters of Glyceraldehyde 3-Phosphate Dehydrogenase.[a]

Condition	$s^0_{20,w}$ (S)	k (l./g)
Holoenzyme	7.79 ± 0.03	0.009 ± 0.001
Holoenzyme + DPN[b]	7.89 ± 0.06	0.010 ± 0.002
Apoenzyme	6.56 ± 0.06	0.007 ± 0.002
Apoenzyme + DPN[b]	6.90 ± 0.05	0.008 ± 0.001

[a] Data were fit by least squares to the equation $s_{20,w} = s^0_{20,w}(1 - kC)$. Plus and minus values are standard errors. The buffer is the same as the Tris buffer of Table I. Temperature was 5°. [b] The ratio of DPN to D-glyceraldehyde 3-phosphate dehydrogenase was held constant at 40 moles of DPN/144 × 10^3 g of protein.

To determine the sedimentation coefficient of the enzymatically active form of D-glyceraldehyde 3-phosphate dehydrogenase, experiments were conducted using the band sedimentation technique of Cohen and Hahn (1965). In order to make the correlation between the moving-boundary sedimentation velocity experiments, the sedimentation equilibrium experiments, kinetic measurements, and band sedimentation velocity experiments, the latter were carried out at both 20 and 5.5°. Further, to ensure that the fast edge of the zone was not contributing the optical density at 340 mμ, the experiments were performed at a variety of loading concentrations of enzyme (0.004–0.92 g/l.). Sedimentation coefficients were calculated from the absorption data according to the method of Cohen and Hahn (1965) and an "*s vs. C*" graph constructed. Such a graph constitutes a type of phase diagram since it is initially flat and gradually curves upward. We chose the flat portion as the true value of the sedimentation coefficient. The rising portion is due to the forward edge of the gaussian band converting most of the DPN into DPNH and tends to yield spuriously large sedimentation coefficients because the band broadens so rapidly (Cohen and Hahn, 1965). Points above 0.08 g/l. were discarded from the 20° data and points above 0.14 g/l. were discarded from the 5.5° data. The average sedimentation coefficients of the remaining points were 8.07 ± 0.18 S at 20° and 7.93 ± 0.25 S at 5.5°. Clearly these values correspond closely to the sedimentation coefficients determined for the native enzyme by the moving-boundary method. It may be concluded that the tetramer of D-glyceraldehyde 3-phosphate dehydrogenase is the active species.

Finally several band sedimentation velocity experiments were performed in *p*-nitrophenyl acetate solutions. Extreme difficulties due to convection and the slow turnover of the enzyme were encountered. These problems were overcome by performing the experiments in 5% sucrose and enzyme concentrations in the band of the order of 5 g/l. The sedimentation coefficient was $s_{20,w}$ = 6.6 ± 0.3 S, a value which implies that both the dimeric and tetrameric species of D-glyceraldehyde 3-phosphate dehydrogenase are active as an esterase. However, the data were not sufficiently precise or well controlled to determine whether the tetrameric species is inactive: the difficulties encountered did not allow a range of loading concentration to be covered.

Discussion

As pointed out by Seery *et al.* (1967) the existence of a dimer–tetramer relation places constraints on molecular weights. In the experiments reported here, this constraint was utilized to find the best dimeric molecular weight. Chemical equilibrium is not prerequisite for a "search" method such as employed in this study. Data from one channel at a time can be calculated by variation of M_1 using eq 1–3 to obtain the best M_1. For example, phosphorylase *a* dissociates slightly in solution while phosphorylase *b* associates slightly. The dissociation of phosphorylase *a* makes the determination of the molecular weight of the tetramer species much more difficult. This difficulty can be overcome by adding phosphorylase *b* to the preparation of phosphorylase *a* to make approximately 1:1 weight ratio of the two molecules. If the search method is then used on the sedimentation equilibrium results, the molecular weight of both phosphorylases *a* and *b* can be determined with greater precision than either independently. Of course, the equilibrium constant obtained in such an analysis is meaningless unless hybrid formation occurs.

The same procedure can be used for more complicated systems of macromolecules but as the number of physical species increases, the resolution of the best M_1 decreases.

The determination of equilibrium constants in highly associated systems by sedimentation equilibrium leads to large errors in the calculated equilibrium constants (Teller *et al.*, 1968). This is easily seen from eq 1–3 of this paper. As the observed molecular weight moments approach the molecular weight of the dimer, the value of k_2 becomes very large. The numerator of these expressions remains finite while the denominator tends to zero. In this study we have attempted to circumvent this

difficulty by the calculation of point-average association constants together with an averaging system to obtain the best constant. To a large degree this has been successful. As noted above the dimer–tetramer equilibrium is normally characterized at pH 7.0 by an association constant of $2.0 \pm 0.5 \times 10^6$ l./mole based on a dimeric molecular weight of 72×10^3 g/mole. Values of observed constants as low as 1.4 and as high as 2.8×10^6 l./mole are included in this category. Because of the presence of varying amounts of heavy material (probably octamer), the constant of 2×10^6 l./mole may well be too high. Evaluation of the data from the experiment in the 30-mm centerpiece gave $k_2 = 1.4 \times 10^6$ l./mole which is probably closer to the value of the true thermodynamic equilibrium constant.

There are three reasons for believing that the dimer–tetramer equilibrium is perturbed by the addition of DPN and phosphate. First, the effect is reproducible giving essentially identical equilibrium constants in experiments done 8 months apart. Second, DPN in Tris-HCl buffer in the absence of added phosphate has no effect upon the observed equilibrium constant. Third, phosphate alone has no effect upon the observed equilibrium constant. The fact that DPN and phosphate affect the dissociation while DPNH with phosphate does not may be correlated with the tighter binding of DPN than DPNH to the enzyme (Velick, 1958).

Malhotra and Bernhard (1968) concluded on the basis of kinetic studies that the enzyme behaved as an $\alpha_2\alpha_2'$ tetramer; however, identical subunits have been reported by Harris and Perham (1965). A structure which is compatible with these results as well as the experiment which we have performed is the type A as opposed to type B.

A B

The effect of adding glyceraldehyde to DPN and phosphate is also reproducible and specific. In this case the system consists of a mixture of dimer and tetramer which are not in chemical equilibrium. This implies that some component of the system is irreversibly coupled to the dimeric form of the enzyme, as verified by the activity studies. The requirement for DPN in the reaction suggests that 1-phosphoglyceric acid may be the reactive agent. The irreversible dimer is almost certainly the ϵ-aminoglyceryl enzyme. The work of Mathew *et al.* (1967) did not show acyl transfer from cysteine to lysine (S→N acyl transfer) at pH 7; however, the duration of our experiments is 20 hr while those of Mathew *et al.* were only a few minutes. The irreversible activity decrease observed in a parallel sample supports this hypothesis. In the control (glyceraldehyde and phosphate) the mole fraction of dimer is 0.23. At 20 hr at 5° in the ultracentrifuge in the presence of all three substrates the enzyme had a dimeric mole fraction of approximately 0.55. This fraction is composed of both reversible dimer (0.18) and irreversible dimer (0.37), where these numbers are based on the assumption that each *N*-acyl tetramer dissociates irreversibly. From the activity loss in the parallel sample of 50%, we would expect the mole fraction of irreversible dimer to be 0.50 on this same assumption. These values are sufficiently close to suggest a 1:1 correlation between inactivation and dimerization, but this conclusion is tentative. Experiments are presently in progress to determine this relation.

p-Nitrophenyl acetate appears to be a specific perturbant of the dimer–tetramer equilibrium. In this case dimers, tetramers, and octamers are present in the solution. We have not tested whether these components are in chemical equilibrium. Here again the ϵ-aminoacyl enzyme is probably responsible for this behavior (Mathew *et al.*, 1967). The active enzyme sedimentation velocity studies performed on this system definitely indicate that the dimer–tetramer equilibrium is shifted in *p*-nitrophenyl acetate solutions. Since this effect is rapid, it may be concluded that the principle influence of this reagent is on the dimer–tetramer equilibrium. Later effects of S→N transfer (Mathew *et al.*, 1967) might be expected to lead to irreversibility as observed for glyceraldehyde. The sedimentation coefficient of the tetramer is about 7.9 S while the active enzyme sedimentation in *p*-nitrophenyl acetate gave $s_{20,w}$ values of 6.6 S. We conclude that the dimeric species is active as an esterase. We are unable to make an unequivocal statement about the esterase activity of the tetramer. Taken at face value, the 6.6S sedimentation coefficient indicates that it is active. However, the principal errors of this method lead to calculation of sedimentation rates which are too large rather than too small.

The active enzyme sedimentation velocity experiments leave little doubt that the tetrameric enzyme is the only active dehydrogenase species. At both 5.5 and 20° the observed sedimentation coefficient was 7.9 S. At the lowest concentration employed (0.004 g/l.), the initial weight per cent of dimer would be 84% at 5°. The solution is further diluted by sedimentation and diffusion. At the highest concentration utilized the initial fraction of dimer was 30%. If both dimer and tetramer were active, we should have observed an apparent concentration dependence of the sedimentation coefficient in these experiments. This was not observed. We conclude that during the interaction of the enzyme with its substrates, the binding of dimeric units to one another becomes much stronger. This result is compatible with the preliminary report by Constantinides and Deal (1968), but is contrary to the results of Agatova (1967).

The studies with apoenzyme both in sedimentation velocity and short-column sedimentation equilibrium clearly indicate that initially the system consists of dimers and tetramers. In view of the observation that only a single, symmetrical boundary was observed in the sedimentation velocity experiments, in all probability the system consists of a chemical equilibrium. The sedimentation equilibrium experiments in 2.5-mm columns, although not reproducible, indicated the presence of monomer and heavy aggregate. From these studies we conclude that upon formation of apoenzyme, the dimer–tetramer equilibrium is shifted in favor of the dimeric form. At later stages, irreversible denaturation occurs.

The final question which arises relates to the contradiction between the results presented here and those reported by Jaenicke *et al.* (1968). These authors have con-

cluded that the holoenzyme does not dissociate. There are two major possibilities for this discrepancy. First, the source of the enzyme was different and, different preparations will almost certainly vary with respect to the degree of dissociation (Teller *et al.*, 1968). Second, most of the results of Jaenicke *et al.* (1968) were obtained at enzyme concentrations greater than 0.5 g/l. From $k_2 = 2 \times 10^6$ l./mole we may calculate that at 0.5 g/l. the molecule is only 17% dissociated at pH 7 and 5°. This would yield a weight-average molecular weight of 132×10^3 g/mole, a value almost within the error limits observed by Jaenicke *et al.* (1968). Reference to the figures of Jaenicke *et al.* (1968) shows that all of the concentration dependent parameters measured for the rabbit muscle enzyme are smaller than those observed for the yeast enzyme, perhaps indicating the existence of a dimer–tetramer equilibrium. Finally, at a very low concentration, errors in molecular weight measurements tend to obscure dissociation when it occurs to only a very slight degree as in this case.

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References

Adams, E. T., Jr., and Fujita, H. (1963), *in* Ultracentrifugal Analysis in Theory and Experiment, Williams, J. W., Ed., New York, N. Y., Academic, p 119.

Agatova, A. I. (1967), *Biokhimia 32*, 1107.

Cohen, R., and Hahn, C. (1965), *Compt. Rend. 260*, 2077

Constantinides, S. M., and Deal, W. C. (1968), *Fed. Proc. 27*, 522.

Creeth, J. M., and Knight, C. G. (1965), *Biochim. Biophys. Acta 102*, 549.

Dandliker, W. B., and Fox, J. B., Jr. (1955), *J. Biol. Chem. 214*, 275.

Elias, H. G., Garbee, A., and Lamprecht, W. (1960), *Z. Physiol. Chem. 319*, 22.

Elodi, P. (1958), *Acta Physiol. Acad. Sci. Hung. 13*, 199.

Fox, J. B., Jr., and Dandliker, W. B. (1956a), *J. Biol. Chem. 218*, 53.

Fox, J. B., Jr., and Dandliker, W. B. (1956b), *J. Biol. Chem. 221*, 1005.

Ginsburg, A., Appel, P., and Schachman, H. K. (1956), *Arch. Biochem. Biophys. 65*, 545.

Harrington, W. F., and Karr, G. M. (1965), *J. Mol. Biol. 13*, 885.

Harris, J. I., and Perham, R. N. (1965), *J. Mol. Biol. 13*, 876.

Jaenicke, R., Schmid, D., and Knof, S. (1968), *Biochemistry 7*, 919.

Kochman, M., and Rutter, W. J. (1968), *Biochemistry 7*, 1671.

Malhotra, O. P., and Bernhard, S. A. (1968), *J. Biol. Chem. 243*, 1243.

Mathew, E., Meriwether, B. P., and Park, J. H. (1967), *J. Biol. Chem. 242*, 5024.

Nichol, L. W., Bethune, J. L., Kegeles, G., and Hess, E. L. (1964), *Proteins 2*, 309.

Park, J. H., Meriwether, B. P., Clodfelder, P., Cunningham, L. W. (1961), *J. Biol. Chem. 236*, 136.

Rao, M. S. N., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724.

Seery, V. L., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6*, 3315.

Sigma Technical Bulletin (1961), Preparation of DL-Glyceraldehyde 3-Phosphate Solution from Diethylacetal (Barium) Salt, St. Louis, Mo., Sigma Chemical Co., No. 10.

Svensson, H. (1954), *Opt. Acta 1*, 25.

Svensson, H. (1956), *Opt. Acta 3*, 164.

Taylor, J. F., Lowrey, C., and Keller, P. J. (1956), *Biochim. Biophys. Acta 20*, 109.

Teller, D. C. (1967), *Anal. Biochem. 19*, 256.

Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1968), *Ann N. Y. Acad. Sci.* (in press).

Velick, S. F. (1955), *Methods Enzymol. 1*, 401.

Velick, S. F. (1958), *J. Biol. Chem. 233*, 1455.

Velick, S. F., Hayes, J. E., Jr., and Harting, J. (1953), *J. Biol. Chem. 203*, 527.

Vinograd, J., Bruner, R., Kent, R., and Weigle, J. (1963), *Proc. Natl. Acad. Sci. 49*, 902.

Yphantis, D. A. (1960), *Ann N. Y. Acad. Sci. 88*, 586.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Yphantis, D. A., and Roark, D. E. (1968), *Ann. N. Y. Acad. Sci.* (in press).