A Disproportionation Mechanism for the All-or-None Dissociation of Mercurial-Treated Glyceraldehyde Phosphate Dehydrogenase*

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ABSTRACT: Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase has been shown to be dissociated from its native tetrameric state into subunits when treated with the mercurial, *p*-mercuribenzoate (PMB). At pH 8 this reaction could be reversed fully by dithiothreitol to regenerate enzymically active tetramers. Hybridization studies employing the native enzyme and a succinylated derivative showed that the addition of PMB led to the formation of monomers and dimers and their amount as a function of time provided an estimate of the rate of protein dissociation following reaction with PMB. Sedimentation experiments indicated that the monomeric species were present in low concentrations and that the PMB-reacted protein existed principally as a mixture of dimers and tetramers in rapid equilibrium. In all solutions of enzyme to which less than stoichiometric amounts of PMB were added there were, after 1 hr, both dissociated molecules which had fully reacted with PMB and native molecules to which no PMB was bound. Thus the process was all or none with respect to the reaction of 14 (±1) SH groups/molecule. The kinetics of mercaptide-bond formation indicated, however, at least two classes of thiol groups in the enzyme which differed considerably in their reactivity toward PMB. The apparent paradox in these results was explained in terms of a disproportionation mechanism of the type first suggested by Szabolcsi, G., Biszku, E., and Sajgo, M. ((1960), *Acta Physiol. 17*, 183). All protein molecules react rapidly with PMB to the extent of one per polypeptide chain, thereby yielding fully inactivated molecules. Either at that stage, or subsequent to reaction of an additional SH group per chain, dissociation occurs. Upon incubation of such partially reacted, inactive molecules disproportionation occurs to yield fully reacted subunits and native, active tetramers. The extent of reactivation is directly related to the fraction of the molecules existing as tetramers after disproportionation, which in turn is dictated by the amount of PMB relative to enzyme. The disproportionation process is pH dependent. At pH 6.4 all of the molecules react with up to 7 moles of PMB to give stable but inactive tetrameric species. When the pH of such solutions is raised to 8, disproportionation occurs, again resulting in all-or-none dissociation. Similar experiments with the apoenzyme showed that although it also appeared to undergo all-or-none dissociation, the cofactor NAD was required for the formation of an enzymically active tetramer following disproportionation.

Studies of the kinetics and extent of reaction of the side chains of the amino acid residues in proteins have contributed substantially to our understanding of the structure and function of these biological macromolecules. Among the reagents employed for such investigations *p*-mercuribenzoate (PMB)[1] has played an important role because of its considerable specificity for SH groups of cysteinyl side chains. This reagent has been used first for determinations of the total number of SH groups in proteins (Boyer, 1954, 1958; Webb, 1966). For such studies the reactions are generally performed on protein solution to which a denaturant is added so that the macromolecules are unfolded and the SH groups are accessible. A second type of investigation involving the reaction of PMB with native proteins yields estimates of the number and size of the classes of SH groups of differing reactivity (Swenson and Boyer, 1957). Third, variations in the kinetics of the reaction of PMB with proteins under different conditions have served as a sensitive measure of changes in the conformation of certain enzymes (Gerhart and Schachman, 1968; Takahashi and Westhead, 1971). Finally, PMB and other mercurials have been used to induce dissociation of oligomeric proteins into subunits (Madsen and Cori, 1956; Gerhart and Schachman, 1968; Bucci *et al.*, 1965; Palacian and Neet, 1970). In a growing number of such proteins, the dissociation to subunits has been shown to be all or none with respect to the binding of PMB, in that addition of an amount of PMB which is insufficient to react with all the SH groups of the protein results in a mixture of dissociated species in which all SH groups have bound PMB and native protein molecules to which no PMB is bound.

Three basic mechanisms have been proposed to explain this all-or-none phenomenon. The first is the so-called "zipper" mechanism postulated by Madsen and coworkers for the dissociation of phosphorylase (Madsen and Cori, 1956; Madsen, 1956; Madsen and Gurd, 1956). According to this mechanism the reaction of the first SH group in each protein molecule is assumed to be rate limiting so that when it has reacted with PMB all other SH groups on that molecule react before any react on a second molecule. Dissociation into subunits may accompany the reaction of the first SH group or some later ones; in either case partially reacted mixtures would contain fully reacted subunits and intact, unreacted protein molecules. A second mechanism invokes a preexisting

* From the Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720. *Received July 1, 1971.* This research was supported in part by U. S. Public Health Service Research Grant GM 12159 from the National Institute of General Medical Sciences, and by National Science Foundation Research Grant GB 4810.

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[1] Abbreviations used are: GPDH, glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.1.1.27); PMB, *p*-hydroxymercuribenzoate; DTT, dithiothreitol.

equilibrium between two conformational forms of the protein, only one of which reacts with PMB. The reactive species could be either an isomeric variant of the enzyme or subunits of it. In the latter case dissociation precedes the reaction with PMB. Such a mechanism appears operative in the all-or-none dissociation of hemerythrin (Keresztes-Nagy and Klotz, 1963) by mercurials, since hemerythrin has been shown conclusively to exist in solution as a monomer–octamer equilibrium prior to reaction of its SH groups (Langerman and Klotz, 1969). A third mechanism has been proposed by Szabolcsi and coworkers (1960) to account for the all-or-none reaction of pig glyceraldehyde 3-phosphate dehydrogenase (GPDH) with PMB. Although they did not demonstrate dissociation of the enzyme into subunits, they presented evidence for the all-or-none reaction with PMB and concluded that their results were compatible with a disproportionation mechanism in which intermolecular transfers of mercurials occur subsequent to an initial partial reaction involving all the protein molecules and PMB.

Since considerable knowledge is now available about the quaternary structure of GPDH (Harris and Perham, 1968; Harrington and Karr, 1965), it seemed of interest to reinvestigate the effect of PMB especially in terms of the dissociation of the protein into subunits. Reaction of this enzyme with PMB has been shown to cause total loss of activity (Velick, 1953), displacement of its firmly bound coenzyme, NAD (Velick, 1953), increased susceptibility to proteolytic attack (Elödi and Szabolcsi, 1959), changes in its far-ultraviolet spectral properties (Friedrich and Szabolcsi, 1967), and marked optical rotatory dispersion and viscosity alterations (Elödi, 1960; Listowsky *et al.*, 1965). In addition, numerous other reports have appeared which describe various aspects of the reaction of this enzyme with PMB and some confusion exists with regard to the relationship between PMB reaction and loss of enzymic activity.

This communication describes a study of the effect of the PMB reaction upon the quaternary structure of rabbit muscle GPDH, both at pH 8 and 6.4, together with a kinetic study of the reactivity of the enzyme's SH groups and the relationship between loss of enzyme activity and PMB reaction. It is shown that when the enzyme reacts with 14 (±1) moles of PMB/mole of enzyme at pH 8, the native tetramer is converted into a mixture of monomers, dimers, and tetramers in rapid equilibrium with the predominant species being dimer. In partially reacted mixtures the dissociation was found to be all or none with respect to PMB bound, since the solutions contained native enzyme to which no PMB was bound together with dissociated species which had fully reacted with PMB. However it was shown by PMB-reactivity studies and enzyme kinetic studies that the reaction is not *initially* all or none, only becoming so after a period of incubation. The results therefore confirm the conclusion reached by Szabolcsi *et al.* (1960) that PMB molecules disproportionate among initially partially reacted molecules, leading finally to a mixture of completely reacted, inactive molecules and native enzymatically active molecules. At pH 6.4 there was no disproportionation and no reactivation. The results are discussed in terms of present knowledge of protein structure.

Experimental Section

Materials

Crystalline GPDH samples were obtained commercially from C. F. Boehringer (Mannheim Corp.). Buffered solutions of the enzyme were obtained from the ammonium sulfate suspension by centrifuging in a clinical centrifuge, dissolving the precipitate in the appropriate buffer, and then dialyzing against the buffer. Protein concentrations were determined spectrophotometrically using the specific absorbance coefficient of 1.00 cm^2 mg^{-1} at 280 nm (Fox and Dandliker, 1956) or refractometrically from the areas of schlieren patterns obtained in the analytical ultracentrifuge, due allowance being made for radial dilution occurring during the experiment. Molar concentrations were calculated on the assumption of a molecular weight of 1.44×10^5 for tetramic native GPDH (Harris and Perham, 1968; Harrington and Karr, 1965; Jaenicke *et al.*, 1968).

PMB was obtained as the sodium salt from Sigma Chemical Co. and stored desiccated at 0–4° in a dark environment. Solutions were prepared by dissolving a weighed amount of PMB in a minimal volume of 10^{-2} M NaOH followed by the addition of the desired amount of buffer. Concentrations were checked spectrophotometrically based on the molar extinction coefficient at 232 nm and pH 7 of 1.69×10^4 M^{-1} cm^{-1} (Boyer, 1954).

NAD, DL-glyceraldehyde 3-phosphate (diethyl acetal barium salt), and dithiothreitol were obtained from Sigma Chemical Co.

Methods

Enzyme assays were performed by a slight modification of the method of Velick (1955). The substrate, DL-glyceraldehyde 3-phosphate, was prepared from its diethyl acetal barium salt (Sigma Chemical Co.). Most of the experiments described were performed with a pH 8 buffer containing 0.1 M Tris·HCl and 1 mM Na_2EDTA. The experiments at pH 6.4 were performed in 0.02 M potassium phosphate (2.071 g of KH_2PO_4 and 1.091 g of $K_2HPO_4 \cdot 3H_2O$ per l.)–1 mM Na_2-EDTA. All pH values were measured at room temperature.

The reactivity of PMB with GPDH was measured at 7.7° by the method of Boyer (1954) based on the change in absorbance at 255 nm as a measure of PMB reacted. The PMB was checked for purity by iodometric titration and, when necessary, was recrystallized (Boyer, 1954). The value obtained by Murdock and Koeppe (1964) for the mercaptide extinction ($\Delta\epsilon_{255} = 4.9 \times 10^3$ M^{-1} cm^{-1}) was used, assuming it to be constant above pH 5.6, irrespective of the buffer used, as verified by these workers. For the assays at pH 8, EDTA was found to alter the mercaptide extinction considerably (*cf.* Boyer, 1954); hence buffers were made up excluding EDTA. We assume that the absence of EDTA led to no inconsistencies between the ultracentrifuge and the spectral data. At pH 6.4, however, the enzyme was found to precipitate during the assay in the absence of EDTA and so it was not excluded from the buffers. At this lower pH the complication due to an alteration in absorbance was not observed. For the measurements of PMB reaction with SH groups, 50 μl of enzyme (approximately 7 mg/ml) was added to 0.95 ml of a solution of PMB (2.5×10^{-4} M) in the appropriate buffer. A similar aliquot of buffer alone was added to the PMB in the reference cuvet. The recording of absorbance change was made in a Cary 14 recording spectrophotometer, of which the sample compartment was thermostated at 7.7°. The recording of absorbance was begun within 15 sec after adding the protein.

ApoGPDH was prepared by the method of Murdock and Koeppe (1964). After passage over charcoal the protein had an $A_{280}:A_{260}$ ratio of 1.9–2.0.

In general, all experiments were performed at a low temperature (7.7°). When GPDH reacts with PMB (Velick, 1953;

Boyer, 1958; Szabolcsi *et al.*, 1960), it becomes very labile, precipitating within a short time at room temperature. At low temperatures, however, the fully reacted enzyme was much more stable and no precipitation was observed if experiments were performed within 3- to 4-hr reaction. Upon storage of such solutions overnight, precipitation did occur even at refrigerated temperatures.

Succinylation of GPDH. Samples of GPDH were reacted with succinic anhydride (Eastman Organic Chemicals) in the following manner. The succinic anhydride was dissolved in dioxane and aliquots were added to the protein solution (approximately 8 mg/ml) which had been dialyzed against 0.05 M Tris·HCl–1 mM Na_2EDTA (pH 8) and to which had been added a 100-fold molar excess of NAD in the same buffer. The volume of the aliquot was such that the concentration of the dioxane did not exceed 5–10% (v/v) in the final mixture and the temperature of the solution was maintained at 0°. With the amounts of succinic anhydride used it was found unnecessary to add NaOH to maintain the pH at 8. The solutions were then incubated at 0° for approximately 60 min after which time the succinic anhydride is depleted (Habeeb *et al.*, 1958). The mixture was then either dialyzed or used as desired.

Zone Electrophoresis. Experiments were performed on cellulose polyacetate strips (14.6 cm, Gelman Sepraphore III) in a Microzone electrophoresis cell (Model R-101, Beckman–Spinco). The electrophoresis buffer contained 0.02 M potassium phosphate and 1.0 mM Na_2EDTA at pH 7.0, precooled to 4°. At this pH native GPDH had a slight mobility toward the anode. Samples were applied to the gel and electrophoresis was conducted for 15 min at a voltage of 250 V (18 V/cm).

Following electrophoresis the positions of the protein bands on the gel were determined by fixing and staining the gel strips in a solution of Ponceau S in trichloroacetic acid and sulfosalicylic acid (Beckman) for approximately 7 min. The membrane was then rinsed in 5% acetic acid and stained further by overnight immersion of the gel in 0.002% nigrosin (Allied Chemical Co.) in 2% acetic acid. The membrane was finally dried after rinsing in 5% acetic acid.

In some cases the enzyme bands were detected by staining for enzyme activity by the method of Lebherz and Rutter (1967) as modified by Meighen and Schachman (1970a).

Sedimentation Studies. All experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with a schlieren optical system and an automatic split-beam photoelectric-scanning optical system (Schachman and Edelstein, 1966). Double-sector cells with aluminum-filled epoxy centerpieces and quartz windows were routinely used for both schlieren and absorption optics. Often two schlieren patterns were recorded simultaneously, one above the other, by using 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. When such pairs of cells were used a reference position on the plate was obtained from the reference slot in the rotor. The temperature was measured and controlled by the RTIC unit supplied by the manufacturer. Sedimentation velocity experiments were performed at 60,000 rpm. When the schlieren optical system was used, patterns were recorded on Metallographic plates and measured with a Gaertner microcomparator. Sedimentation coefficients of individual species were determined from the rate of movement of the sedimenting boundary as measured by the position of the maximum ordinate of the schlieren pattern. Weight-average sedimentation coefficients (Goldberg, 1953) were measured as the rate of movement of the position of the square root of the second moment of the refractive index gradient curve. Usually 20–40 coordinates on each schlieren pattern were measured relative to the solvent base line in order to calculate this position. In most experiments, plots of the logarithm of the square root of the second moment *vs.* time were essentially linear and the weight-average sedimentation coefficients ($s_{20,w}$) were calculated from the slope of the straight line fitted through all the points by the method of least squares. In a few cases a departure from linearity was observed due to a shift in the position of chemical equilibrium caused by radial dilution during sedimentation. In such cases the $s_{20,w}$ values were calculated from the slope of the tangent to the curve and the corresponding protein concentration was calculated from the amount of radial dilution known to have occurred. Calculations of $s_{20,w}$ were made with the aid of a computer program written for the purpose. All sedimentation coefficients were corrected to values corresponding to a solvent with the viscosity and density of water at 20° (Svedberg and Pedersen, 1940). Solvent densities were determined pycnometrically and a partial specific volume of 0.73 ml/g was used for both native and modified enzyme at 7.7° (Taylor and Lowry, 1956). The molecular weight of the labile PMB-treated enzyme was measured by the Archibald (1947) procedure according to the method of Klainer and Kegeles (1955). The rotor speed was 12,000 rpm and only measurements made at the meniscus were used for the determination of weight-average molecular weights. The initial concentration was determined refractometrically in a separate experiment with a synthetic boundary cell.

In addition to the weight-average sedimentation coefficient determinations, the proportion of undissociated material in mixtures was estimated from area measurements of schlieren patterns. This was performed both by trapezoidal integration of values read on the microcomparator and by planimeter measurements of enlarged traces of the schlieren patterns. Agreement between the two methods was good.

Experiments employing the split-beam photoelectric scanning optical system were performed with light of 280 and 248 nm, scans at each of these wavelengths being taken in quick succession. Recorder deflections in a vertical direction were determined for each trace and converted to absolute absorbance values using calibration curves constructed from separate experiments performed at the respective wavelengths on solutions of known optical density.

Results

Dissociation of GPDH by PMB. In Figure 1 are shown representative sedimentation velocity patterns obtained with increasing amounts of PMB relative to the enzyme (7.8 g/l.) at pH 8 and 7.7°. Clearly, in the time between PMB addition and ultracentrifugal analysis, the enzyme is dissociated to slower sedimenting material, the amount of which increases as the amount of PMB added is increased. In order to calculate the stoichiometry of the dissociation with respect to the amount of PMB added, we measured the area corresponding to the faster sedimenting species (which was shown to have an $s_{20,w}$ characteristic of native enzyme). Figure 2 shows the results of these measurements as the percentage of undissociated GPDH plotted as a function of moles of PMB per mole of enzyme. Because of the difficulty in measuring the areas of the overlapping boundaries for the samples containing more than 8 moles of PMB/mole of enzyme, the $s_{20,w}$ values of all the samples were measured and also plotted against the amount of PMB added (Figure 3). It is seen from both Figures

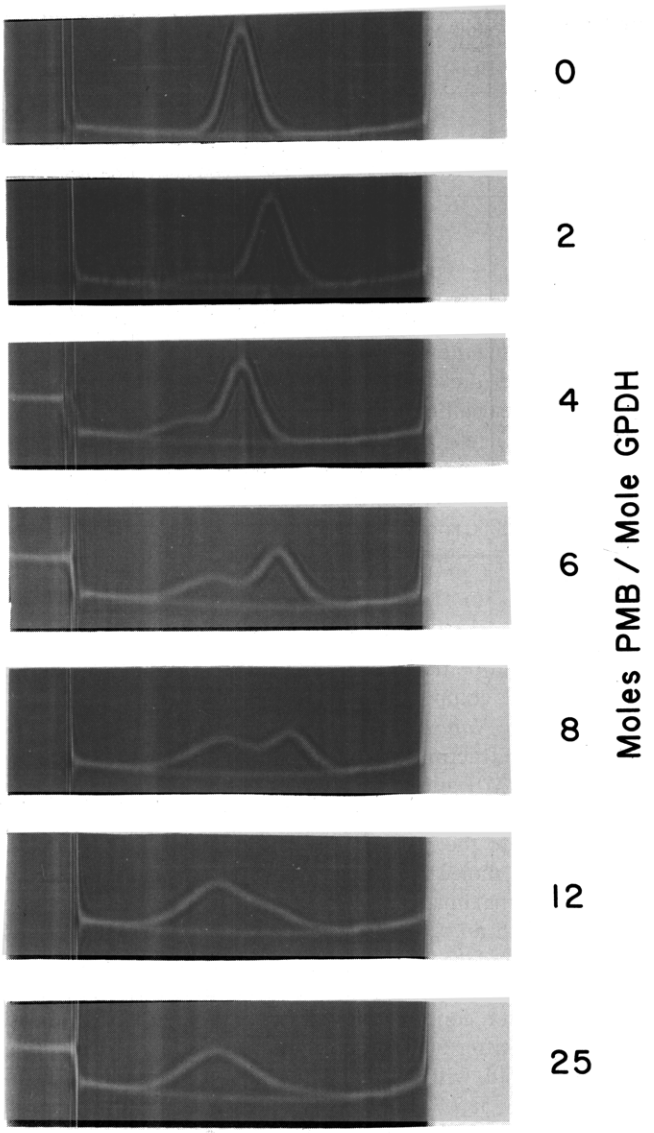

FIGURE 1: Sedimentation velocity patterns of GPDH treated with various molar ratios of PMB to enzyme. The photographs were taken with a phase-plate angle of 70° after approximately 1-hr sedimentation at 60,000 rpm. Movement is from left to right. The protein concentration was 7.8 mg/ml, and the buffer was 0.1 M Tris·HCl–1 mM EDTA, pH 8, 7.7°.

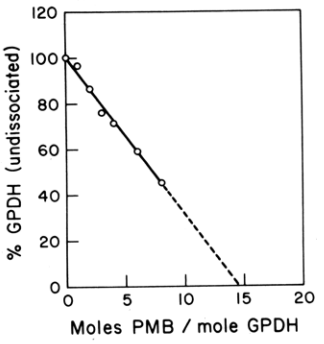

FIGURE 2: The areas of the faster sedimenting boundary (7.2 S), shown in Figure 1, were calculated both by trapezoidal integration of coordinates read on a Gaertner microcomparator and from planimeter measurements of enlarged traces of the schlieren patterns. The areas obtained in each case agreed well, were corrected for radial dilution, and were then expressed as a percentage of the area obtained for a GPDH sample to which no PMB had been added. The per cent undissociated GPDH on the ordinate was plotted against the molar ratio of PMB to enzyme. The line represents an averaging of the data by the method of least squares.

2 and 3 that the extent of dissociation of GPDH is directly proportional to the amount of PMB added. The solid lines in Figures 2 and 3 have been fitted to the data by the method of least squares and show that maximal dissociation occurs upon reaction of 14 (±1) molecules of PMB per molecule of GPDH.

Nature of the Dissociated Material. From Figure 1 it is seen that the material formed upon dissociation of GPDH by PMB gives a broad sedimenting boundary and presumably does not represent a single, homogeneous, protein species. Indeed, the $s_{20,w}$ value measured for this material (4.9 S) is higher than expected for the dimer of native GPDH at this concentration. It seemed likely, therefore, that the broad boundary was in fact a reaction boundary, representing primarily a mixture of dimers and tetramers in equilibrium. Hybridization studies, performed as described later in this paper, showed that monomers are also formed by the dissociation but apparently the monomer–dimer association constant is large. The measured weight-average sedimentation coefficient, $s_{20,w}$, would therefore represent the average sedimentation coefficient of all the individual molecules in the plateau region, weighted according to the amount of each present at the particular protein concentration at which it was measured. That the rate of chemical equilibration of the various species is in fact rapid, compared to the rate of sedimentation, is indicated by the fact that only a single, albeit asymmetric, boundary was observed for the fully reacted material (Gilbert, 1959). The series of schlieren patterns shown in Figure 1 must therefore represent a boundary which is due to undissociated GPDH superimposed upon a spread reaction boundary corresponding to material which has been dissociated by PMB. To test this hypothesis further, we measured $s_{20,w}$ values of enzyme which had been fully reacted with PMB (16 moles of PMB added per mole of GPDH) and sedimented at a series of different initial enzyme concentrations. In each case a single, asymmetric, boundary was obtained. Figure 4 is a plot of the results in terms of the weight-average sedimentation coefficient as a function of protein concentration. In order to curve fit these data it is necessary to know the

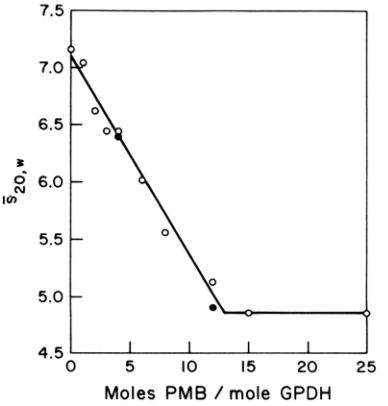

FIGURE 3: A plot of $\bar{s}_{20,w}$ of PMB-dissociated enzyme *vs.* the amount of PMB added per mole of GPDH. The $\bar{s}_{20,w}$ values were calculated from a series of exposures taken during the sedimentation velocity experiments described in Figure 1. The solid points (●) represent additional experiments performed on solutions containing a 100-molar excess of NAD over protein.

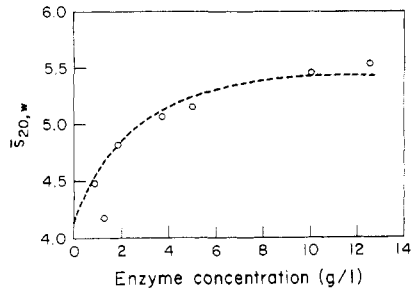

FIGURE 4: A plot of $s_{20,w}$ *vs.* protein concentration for a GPDH sample dissociated at pH 8, 7.7°, by the addition of 16 moles of PMB/mole of enzyme. The various concentrations were obtained by a serial dilution of the most concentrated sample (12.5 mg/ml) which had been reacted with PMB. The samples were sedimented at 60,000 rpm.

TABLE I: Weight-Average Molecular Weights of PMB-Dissociated GPDH.[a]

Time of Sedimentation (min)	Concn at Meniscus (g/l.)	$M_{w,app}$ ($\times 10^{-3}$)	Association Constant (l./g)
40	5.2	97	0.15
48	4.9	99	0.19
56	4.8	99	0.19

[a] The apparent weight-average molecular weights ($M_{w,app}$) were calculated at different times from schlieren patterns photographed during an Archibald sedimentation experiment. The association constants were calculated for a dimer (M = 72,000)–tetramer (M = 144,000) equilibrium reaction, assuming negligible amounts of monomer in the system. The experiment was performed at pH 8, 7.7°, with an angular velocity of 12,000 rpm.

values of the equilibrium constants relating the concentrations of the various molecular species. Unique values of these constants cannot be obtained from the data shown in Figure 4, however, because the number of parameters determining the sedimentation coefficient of an interacting system is too large. For this reason it was decided to estimate the equilibrium constants by determining the weight-average molecular weight of a solution of GPDH which had been fully reacted with PMB. Because of the lability of such a mixture the method of choice was the Archibald (1947) procedure, which yields values of the molecular weight within 1–2 hr after preparation of the solution.

A sample of GPDH (7.4 g/l.) was dissociated by adding a 16-fold molar excess of PMB and was sedimented at 12,000 rpm, which was shown to be a suitable speed for the Archibald procedure. From a series of three schlieren patterns the concentrations of protein at the meniscus and the corresponding weight-average molecular weights were calculated. The values are shown in Table I. From these molecular weights an association equilibrium constant (about 0.18 l./g) was calculated, assuming a dimer (M = 72,000)–tetramer (M = 144,000) equilibrium.

As a test of this equilibrium constant in terms of the $s_{20,w}$ *vs.* concentration data (Figure 4), the latter have been fitted in terms of a dimer–tetramer equilibrium. For this curve fitting we employed the above equilibrium constant (0.18 l./g), and $s_{20,w}$ for tetramer equal to that of native GPDH ($s^0_{20,w}$ = 7.79 S; Hoagland and Teller, 1969), a concentration dependence of $s_{20,w}$ for both dimer and tetramer equal to that of native GPDH (k = 0.008 l./g; Meighen and Schachman, 1970a; Hoagland and Teller, 1969), and an $s^0_{20,w}$ for dimer of 4.13 S. The latter value gave a better fit of the data than the value of $s^0_{20,w}$ = 4.90 S which would be expected solely on the basis of the reduced molecular weight as calculated from the Scheraga–Mandelkern equation (Scheraga and Mandelkern, 1953; Schachman, 1959). The line generated by these parameters is presented in Figure 4. Clearly this model is a reasonable approximation of the data confirming that the *principal* components in a mixture of PMB-dissociated GPDH are dimeric and tetrameric species in rapid equilibrium. Hence, although the presence of monomer in the system is shown by the hybridization data (see below) we concluded that its concentration must be low relative to the total protein concentration, presumably because its association constant (to dimer) is large. The system could be described more precisely only if the exact monomer–dimer equilibrium constant could be determined and if the values of $s^0_{20,w}$ and k for the various components were known.

All-or-None Nature of the Dissociation. The proportionality between the fraction of undissociated molecules and the amount of PMB added (Figures 1–3) made it of interest to determine whether in fact the dissociation was all or none with respect to the number of PMB molecules reacting with each enzyme molecule, *i.e.*, whether the dissociated material had reacted maximally with PMB and the faster sedimenting material (with an $s_{20,w}$ corresponding to native GPDH) was devoid of bound material.

Various molar amounts of PMB were added to samples of the enzyme at a concentration of approximately 8 mg/ml and the mixtures after 30–60 min at approximately 7° were then diluted tenfold with pH 8 buffer and subjected to ultracentrifugation at 7.7°. The distribution of material in the cell was measured first by scanning for transmitted light of wavelength 280 nm and then immediately afterward with light of 248-nm wavelength. Since neither PMB nor its mercaptide absorbs significantly at 280 nm, the scan at this wavelength provides essentially a measure of the distribution of protein alone, whereas that at 248 nm is a measure of the concentration of the PMB mercaptide plus any unreacted protein and PMB (Gerhart and Schachman, 1968). From these measurements, the relative absorbance at 280 and 248 nm (A_{280}:A_{248}) was calculated for the dissociated protein in each sample. The ratio was constant across the boundary. The results are tabulated in Table II, together with the A_{280}:A_{248} value of a sample of fully dissociated enzyme (14 moles of PMB/mole of GPDH) which was determined in a spectrophotometer.

The corresponding ratio for undissociated protein could not be measured directly because of the previously described reaction boundary which prevented the formation of a *plateau* of dissociated enzyme and which therefore precluded subtraction of the absorbance due to dissociated enzyme from the total absorbance of the mixture. This ratio could be calculated indirectly, however, since for each solution the total optical density at 280 and 248 nm was measured and the percentage of dissociated protein could be estimated from Figures 2 and 3. Then, knowing that PMB and its mercaptide have negligible absorption at 280 nm, and using the value of A_{280}:A_{248} for the dissociated enzyme, obtained as described above, we calculated the values of A_{280} and A_{248}, and their

ratio, for the undissociated protein. The values obtained for each solution are tabulated in Table II, together with that for native GPDH.

In order to show that these results were not anomalous due to dilution of the samples following the initial reaction with PMB, we performed additional experiments in which the PMB was added *after* diluting the samples. The results, for molar excesses of PMB over enzyme of 4, 8, and 12, are included in Table II. Clearly, the results are independent of the concentration of enzyme over the range examined.

It is apparent that the dissociated material in each sample had a ratio of $A_{280}:A_{248}$ equal to that for fully reacted material (0.52) and the undissociated enzyme had a ratio equal to that measured separately for native enzyme (1.3). In each case the ratios were independent of the total amount of PMB added. Theoretically expected ratios have also been included in Table II for the binding of 1–3 moles of PMB/mole of native GPDH. These calculations were based on molar extinction coefficients at 248 and 280 nm for a mercaptide linkage of 1.4×10^4 and 0.1×10^4 M^{-1} cm^{-1}, respectively (Boyer, 1954), and the value for the enzyme listed under materials. It can be seen that the value (1.17) expected for the binding of only one PMB per molecule was below each of the values determined experimentally for the undissociated enzyme. Thus it can be concluded that the results are not consistent even with only one PMB molecule being bound per molecule of undissociated enzyme. The reaction is, therefore, all or none; after the time elapsed between the addition of the mercurial and the ultracentrifugal measurements, there are 14 (±1) PMB molecules bound to each molecule of dissociated GPDH, and none bound to undissociated GPDH.

Reversal of the Dissociation at pH 8. Earlier reports (Velick, 1953) that PMB inhibition of GPDH could be reversed fully by the addition of cysteine suggested that this substance would also reverse the dissociation. At enzyme concentrations of the order of 8 mg/ml, partial reversal was in fact observed by ultracentrifugal analysis; but the results were invariably complicated by appreciable aggregation and precipitation of the protein, even in the presence of relatively low concentrations of cysteine. Presumably cysteine, in addition to displacing bound PMB from some molecules, also accelerates the irreversible aggregation of some protein.

The dithiol compound, dithiothreitol (DTT) (Cleland, 1964), however, was found to reverse the dissociation, both in the presence and absence of small molar excesses of NAD; this occurred without causing a concomitant precipitation of protein, if DTT was added within about 4 hr after the dissociation by PMB. Maximal reversal was obtained with a 200–300 molar excess of DTT and enzyme concentrations of the order of 8 mg/ml. The conversion of PMB-dissociated enzyme to material having a sedimentation coefficient corresponding to native enzyme was a slow process, requiring several hours for completion. Furthermore, 100% reconversion could not be obtained even when the DTT was added within 30 min of the PMB. The extent of the reconversion was of the order of 75% under the above conditions upon overnight standing in DTT at 0–5°. The unreconstitutable material could be precipitated preferentially if the solutions were maintained at room temperature for 2–4 hr or if the pH was adjusted to 6.5, in which case it precipitated immediately.

Some experiments were performed to obtain a *fully* reversible dissociation of the enzyme by PMB. If the enzyme (approximately 8 g/l.) was dissociated by PMB in the presence of a 100-fold molar excess of NAD, complete reconversion to tetramer occurred upon the addition of DTT (0.02 M), urea (0.2 M), and NaCl (0.2 M) and then allowing the solutions to stand overnight at 0–4° followed by dialysis and the addition of a 10-fold molar excess of NAD in order to replace that which had been displaced by the PMB (Velick, 1953). This reassociation caused the boundary to change from one corresponding to fully reacted enzyme (*cf.* bottom boundary, Figure 1) to one corresponding to native GPDH (*cf.* top boundary, Figure 1). The $\bar{s}$ values for such samples are indicated in Figure 3. In a control sample to which urea was not added only 75% of the protein was converted to material with the sedimentation coefficient of the native enzyme.

pH Dependence of the Dissociation of GPDH by PMB. Generally both the extent and rate of reaction of protein SH groups with PMB are pH dependent, both because these groups may exist in ionized and un-ionized states and because they may be masked to different extents by pH-dependent conformational changes in the protein itself (Boyer, 1954, 1958; Webb, 1966). In particular, although rabbit muscle

TABLE II: All-or-None Nature of the Dissociation of GPDH by PMB.[a]

Moles of PMB/ Mole of Protein	% Dissociated	A_{280}/A_{248} Experimental: Dissociated	A_{280}/A_{248} Experimental: Undissociated	A_{280}/A_{248} Theoretical[b]
0	0		1.32	1.32
1				1.17
2				1.06
3				0.97
4	28	0.51, 0.52[c]	1.3	
6	42	0.51	1.4	
7[d]	50	0.50	1.2	
8	56	0.55, 0.45[c]	1.4	
10	69	0.50	1.3	
12	83	0.53, 0.54[c]		
14	100	0.52		

[a] Values of the ratio, $A_{280}:A_{248}$, were obtained for the dissociated and undissociated components after dilution of GPDH samples which had been treated with various molar ratios of PMB. The values for the dissociated material were measured directly during ultracentrifugation at 7.7° and the corresponding ratios for undissociated enzyme were calculated using molar extinction coefficients for mercaptide of 1.4×10^4 at 248 nm and 0.1×10^4 at 280 nm, and assuming a protein molecular weight of 144,000. The experimental procedure is described in the Experimental Section. [b] The theoretical values are those which would be expected if the PMB bound to tetrameric GPDH without dissociating it. The calculated values are based on the molar extinction coefficients listed above. [c] For these samples the reaction with PMB was carried out at the same protein concentration (0.8 g/l.) as for the centrifuge experiments. [d] This particular sample (0.94 g/l.) was reacted with PMB at pH 6.4, at which pH the enzyme is not dissociated by this amount of PMB (Figures 5 and 6). The pH of the solution was then adjusted to pH 8 by the addition of an aliquot of 0.1 M Tris·HCl–1 mM Na_2EDTA (pH 10) and the solution centrifuged and analyzed as in the other experiments.

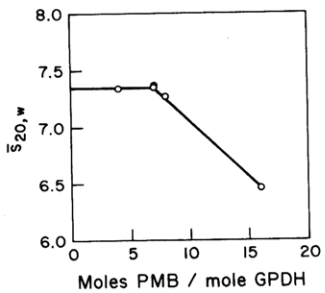

FIGURE 5: A plot of $\bar{s}_{20,w}$ *vs.* PMB added to GPDH samples at pH 6.4, 7.7°. The protein concentration was 8.0 mg/ml and $\bar{s}_{20,w}$ values were calculated from a series of exposures taken during sedimentation experiments at 60,000 rpm.

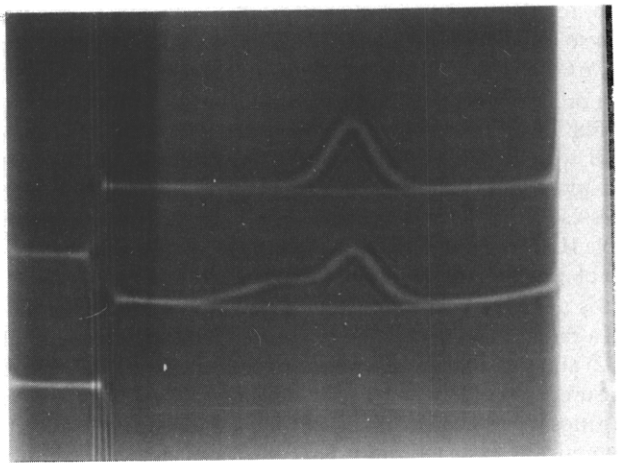

FIGURE 6: Schlieren patterns taken during a single centrifuge experiment of GPDH treated as follows. A sample of GPDH at pH 6.4 was reacted with 7 moles of PMB/mole of enzyme. After 1 hr a small amount of 2 M Tris–1 mM EDTA (pH 10) was added to an aliquot of the PMB-treated GPDH. This addition yielded a pH of 8.0. To a corresponding aliquot was added an equal volume of pH 6.4 buffer. Both samples were then ultracentrifuged at 60,000 rpm, 7.7°, and photographed with schlieren optics using a phase-plate angle of 70°. The upper pattern, taken after approximately 1-hr sedimentation, represents the enzyme at pH 6.4, while the lower one represents that taken to pH 8. The protein concentration was 8.2 mg/ml. Sedimentation is from left to right.

GPDH has not been previously studied at pH 8 its SH groups are known to show a marked pH dependence in the reaction with PMB both in the number of groups reacted and in the rate of their reaction (Murdock and Koeppe, 1964; Boyer and Segal, 1954). It seemed of interest, therefore, to determine whether the dissociation of GPDH by PMB at a lower pH is similar to that observed at pH 8. Because the enzyme becomes highly labile below pH 6 (unpublished observations; Velick, 1955; Cseke and Boross, 1967), experiments at pH 6.4 were chosen for this study.

Experiments analogous to those represented in Figures 1–3 were performed on samples of enzyme previously dialyzed to pH 6.4 and 7.7°. The dependence of $\bar{s}_{20,w}$ on the amount of added PMB is given in Figure 5. At this pH, a total of seven PMB molecules reacted with virtually no dissociation of the enzyme, as evidenced by the fact that only a single boundary was observed in each case with a value of $\bar{s}_{20,w}$ equivalent to that of native GPDH. That SH groups had, in fact, reacted at this pH is shown in the following section. Upon addition of more than 7 moles of PMB/mole, appreciable dissociation does ensue (Figure 5), with the formation of a more slowly sedimenting, diffuse boundary. When the enzyme solution was buffered with imidazole instead of phosphate some dissociation was observed with less than seven PMB molecules per GPDH molecule, although to a much lesser extent than was observed under the equivalent conditions at pH 8. It seems, therefore, that the dissociation at pH 6.4 is somewhat dependent on specific anion effects.

In order to learn more about the mechanism of the dissociation at both pH 8 and 6.4 we reacted a sample at pH 6.4 with 7 moles of PMB/mole of GPDH and then adjusted the pH to 8 by the addition of a small aliquot of 2 M Tris base (containing 1 mM EDTA) at pH 10. A corresponding volume of pH 6.4 buffer was added to a control sample and both were examined with schlieren optics in the ultracentrifuge. The resulting sedimentation velocity patterns are shown in Figure 6. For the control a single boundary was observed with an $s_{20,w}$ of 7.35 S. In contrast the protein which was subjected to PMB treatment at pH 6.4 and followed by an increase of pH to 8 showed two boundaries which were similar to those observed when the PMB dissociation was performed directly at pH 8 (*cf.* Figure 1). Accompanying the increase in pH was a decrease in $s_{20,w}$ from 7.35 to 6.18 S, a value almost equal to that found when the PMB reaction was conducted at pH 8 initially.

Since raising the pH after treatment of GPDH with PMB was accompanied by dissociation of some of the protein it was of interest to determine the distribution of the mercurial among the protein molecules. Hence the pH 8 sample was examined in the ultracentrifuge with the photoelectric scanning absorption optical system and the $A_{280}:A_{248}$ ratios were determined for the fast and slow compounds. As seen in Table II the fast component had a ratio of 1.2, almost equal to that of native GPDH, and the slow component had a ratio of 0.50, a value characteristic of protein which was fully reacted with mercurial. Thus the sample at pH 8 exhibited all-or-none binding of PMB.

In the light of this finding it was important to inquire whether the initial reaction at pH 6.4 was all or none with respect to PMB binding. If it were, then half of the protein molecules would have reacted with 14 PMB molecules and the other half with none. This seems unlikely since the addition of PMB to an amount greater than 7 moles/mole of enzyme causes a decrease in the sedimentation coefficient as shown in Figure 5. The presence of a single boundary when 7 moles of PMB is added per mole of GPDH at pH 6.4 thus indicates that all of the protein molecules had reacted to about the same extent and that the reaction at that pH is not all or none with respect to binding PMB. The reverse reaction, *i.e.*, adjusting to pH 6.4 a GPDH sample reacted with 7 moles of PMB/mole at pH 8, was not feasible since GPDH molecules which had fully reacted with PMB precipitated when the pH was decreased below pH 7. Additional evidence regarding the reactivity of the sulfhydyl groups of GPDH was provided from kinetics studies at pH 6.4 and 8.

Kinetics of PMB Mercaptide Formation at pH 6.4 and 8. The extent and rate of reaction of GPDH with PMB were measured spectrophotometrically at 255 nm by the method of Boyer (1954). The reaction of PMB with GPDH was allowed to proceed for 1 hr at the end of which time the maximum absorbance change appeared to have occurred. During the reaction, less than 17% of the total PMB was consumed. At both pH values the measured absorbance change corresponded to the reaction of approximately 14 SH groups/

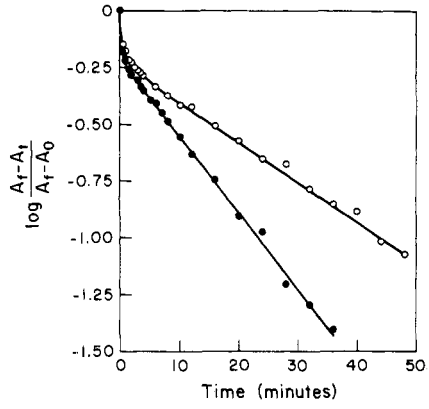

FIGURE 7: Plots representing a pseudo-first-order kinetic analysis of the reaction of PMB with GPDH at pH 6.4 and 8. The values for the absorption at 255 nm were determined at zero time (A_0), at various times during the reaction (A_t), and after the reaction appeared to be complete (A_∞). The symbols, ○ and ●, represent measurements made at pH 6.4 and 8.0, respectively.

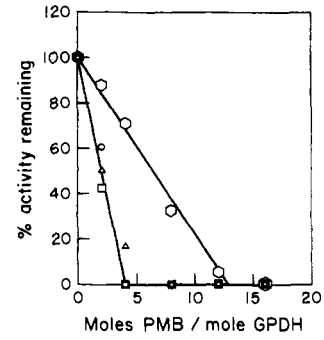

FIGURE 8: Results of assaying GPDH in the presence of different amounts of PMB, both immediately and 1 hr after the addition of PMB. The PMB was added to GPDH samples at 7.7° which were then diluted appropriately into the assay buffer. The experiments were performed both at pH 8 and at 6.4. The symbols, ○ and ○, represent assays at pH 8, at approximately 15 sec and 1 hr, respectively, and □ and △ represent the corresponding assays of samples treated with PMB at pH 6.4 after about 15 sec and 1 hr, respectively. The enzymic activity obtained in each case was calculated relative to that of a similar enzyme sample which was not treated with PMB. The activity of this sample was essentially unchanged during incubation at either pH for 1 hr.

molecule of GPDH (assuming a molar extinction increment at 255 nm of 4.9×10^3 M^{-1} cm^{-1}). Because of the reasonable large excess of PMB in the reaction mixture, the results were treated in terms of pseudo-first-order kinetics; Figure 7 gives plots of log $(A_t - A_t)/(A_t - A_0)$ *vs.* t, where A_t and A_0 represent the final and initial absorbance observed in the experiments and A_t represents the absorbance at time, t.

From Figure 7 it is clear that at both pH 6.4 and 8, the reaction occurs in at least two stages, an initial fast reaction (or reactions) followed by a second and much slower process. At both pH's, the second stage, which is indicated by the linear portion of the pseudo-first-order plot, becomes manifest after the formation of 7 (±1) mercaptide bonds per molecule of GPDH. The formation of the first 7 (±1) bonds was too rapid for a detailed kinetic analysis but the slower phase of the reaction is described by a first-order rate constant of 6.9×10^{-4} sec^{-1} at pH 6.4 and 1.3×10^{-3} sec^{-1} at pH 8. The kinetic pattern, consisting of at least two independent pseudo-first-order reactions and illustrated in Figure 7, has been observed previously by other workers (Szabolcsi *et al.*, 1960; Friedrich and Szabolcsi, 1967) with pig GPDH. Quite clearly, at least two major classes of sulfhydryl groups exist in rabbit muscle GPDH, each class differing considerably in its reactivity toward PMB.

At first sight these kinetic results present a paradox with respect to the all-or-none nature of the PMB reaction since they show that *all* GPDH molecules in the solution must react partially with PMB before any can react fully. Seemingly the results are consistent only with the explanation first proposed by Szabolcsi *et al.* (1960); *viz.*, in mixtures of partially reacted GPDH, a disproportionation of PMB groups must occur between two or more protein molecules such that some molecules acquire a full complement of mercaptide groups and others revert to their native, PMB-free state.

If such a disproportionation occurs, then it may be revealed by studies of the kinetics of loss of enzyme activity, since if PMB binding becomes all or none with respect to protein dissociation, then it may also become all or none with respect to enzyme activity. Hence, experiments were initiated to determine the relationships among enzyme activity, PMB concentration, and time.

Loss of Enzymic Activity upon Reaction with PMB. Enzyme solutions were prepared at concentrations comparable to those used in the sedimentation velocity experiments (approximately 8 mg/ml, pH 8, 7.7°) and to them were added varying amounts of PMB in small volumes of buffer. An aliquot of each solution was immediately diluted with buffer and assayed. Each solution was then incubated at 7.7° for 1 hr and reassayed. The results are shown in Figure 8. It is seen that when the samples were assayed initially, total loss of activity was observed on addition of only 4 moles of PMB/mole of enzyme. However after 1-hr incubation all of the partially reacted samples (with less than 14 moles of PMB/mole of enzyme) had strikingly reactivated such that the per cent activity did parallel the per cent undissociated protein as measured in the ultracentrifuge (Figures 2 and 3). The time of incubation (1 hr) is approximately that which elapsed before the ultracentrifugal observations were made following the addition of the mercurial. Thus after this time a total of approximately 14 moles of PMB is required to inactivate completely 1 mole of enzyme.

These findings are in accord with the results of the kinetic studies of SH group reactivity in showing that the PMB reaction initially is not all or none. The reactivation (to varying extents) of all the samples which are partially reacted with PMB must result from the transfer of groups between two or more enzyme molecules. Although the dependence of reactivation on enzyme concentration was not investigated in detail, it was observed that at least partial reactivation occurred even at concentrations as low as those used for enzymic assay (8.7×10^{-4} g/l.).

The above experiment was repeated with enzyme incubated at pH 6.4, and the results are also presented in Figure 8. Again, total loss of activity is observed upon reaction of 4 moles of PMB/mole of GPDH, but at this pH there is no time-dependent recovery of activity. Presumably at this pH the conformation of the protein, or possibly the fact that the SH groups are not ionized, precludes the disproportionation which occurs at pH 8.

The fact that 7 SH groups react at pH 6.4 without any dissociation of the protein, and disproportionation appears not to occur at this pH, suggested that at pH 8, also, the first 7 SH groups may react without any dissociation of the enzyme.

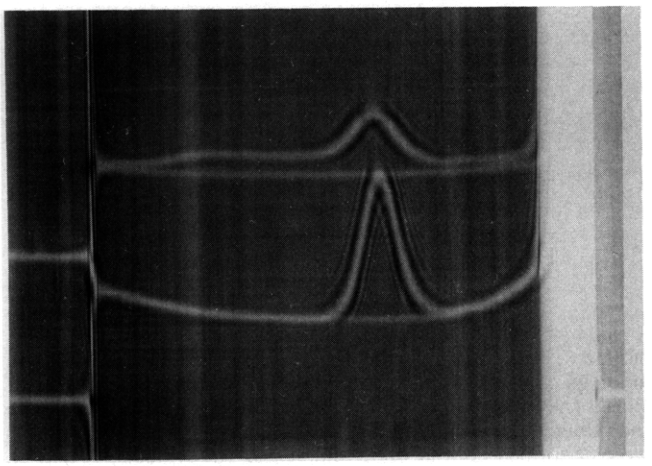

FIGURE 9: The lower schlieren exposure represents a GPDH sample (7.9 mg/ml) which was treated at 0° with 1.5 moles of succinic anhydride/lysine residue on the protein, in the presence of a 100 molar excess of NAD over protein. The upper pattern represents an identical protein sample, treated similarly except that the excess NAD was omitted from the reaction. Each sample was immediately centrifuged at 60,000 rpm at 7.7° and the above exposures were taken with a phase-plate angle of 70°, approximately 1 hr after sedimentation was commenced. Movement is from left to right. Buffer as in Figure 1.

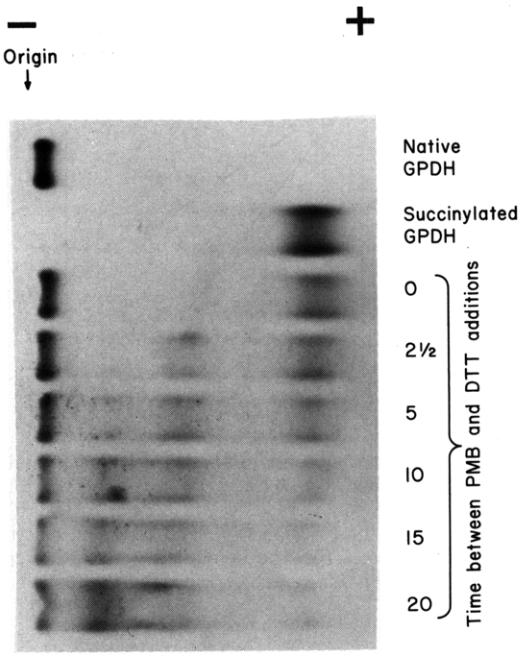

FIGURE 10: Cellulose acetate electrophoresis of the hybrids obtained by adding a 30 molar excess of PMB to mixtures of equal amounts of native and succinylated GPDH. The indicated times (in minutes) refer to the interval between adding PMB and making the solution 0.02 M with respect to DTT, the latter being added to halt any further action by PMB by displacing it from the enzyme molecules. The DTT also converts those subunits present in solution back to tetramers, thereby creating the hybrid sets. After the addition of DTT, samples were left overnight at 0–4°, before carrying out electrophoresis. The positions of the bands on the cellulose acetate were located by staining for protein, as described in the Experimental Section.

In order to relate the dissociation to the rate of reaction of SH groups attempts were made to estimate the rate of dissociation of the enzyme following addition of PMB.

Rate of Dissociation of GPDH by PMB as Measured by Hybrid Formation. If two distinguishable, but similar, variants of an oligomeric protein are available, the subunits of which are interchangeable by a reversible dissociation reaction, then observation of the rate of hybrid formation when the two species are mixed under dissociating conditions may provide a measure of the rate of dissociation of the protein. Meighen and Schachman (1970a) have shown that an electrophoretic variant of GPDH can be obtained by succinylating native GPDH with succinic anhydride and that this modified species does form hybrids with native enzyme when mixed together in dissociating conditions followed by reconstitution. Since it has been shown above that PMB causes dissociation of GPDH and its effect is reversed by DTT, attempts were made to measure the rate of dissociation by adding the PMB to a mixture of native and succinylated enzyme molecules and then adding DTT at successive times to affect reconstitution and the production of hybrid sets. Subsequent electrophoretic separation of the hybrids from the native and succinylated components and measurement of the amount of hybrid formed as a function of the time between PMB and DTT addition would then give a measure of the rate of dissociation of GPDH by PMB. The accuracy in the determination of the rate constant of dissociation would depend on the experimental prodedures used for the separation and measurement of the hybrids formed; for the present study the experiments were designed merely to determine whether dissociation accompanied the reaction of one or the other of the two major classes of PMB-reacting SH groups in the molecule.

In preparing succinylated samples of GPDH, Meighen and Schachman (1970a) had found that the succinylation reaction always produced, in addition to a species which retained its quaternary structure and hybridized with native GPDH, a second, inert, component which was a dissociated derivative of the enzyme. They also observed protein precipitation during the succinylation. We have found that both of these complications are obviated if the succinylation is performed at 0° in the presence of a 100-fold molar excess of NAD over enzyme. The succinic anhydride was dissolved in dioxane before adding it to the enzyme solution. Figure 9 shows a sedimentation velocity pattern of a GPDH sample which had been succinylated in the presence of excess NAD, compared with an identical sample reacted in the absence of NAD. The fast component in each case sedimented with an $s_{20,w}$ of 6.3 S, a value lower than expected for native GPDH, presumably due to the primary charge effect (Pedersen, 1958) brought about by the large charge on the succinylated protein. The sample which had been succinylated in excess NAD also migrated as a single electrophoretic component (Figure 10) whereas that reacted in the absence of NAD showed two bands upon cellulose acetate electrophoresis (*e.g.*, Meighen and Schachman, 1970a). A sample corresponding to the addition of 1.5 moles of succinic anhydride/mole of lysyl residue was chosen for further work. Such a sample retained about 6% of its original activity; a corresponding sample reacted in the absence of an excess of NAD was totally inactivated.

Succinylated GPDH was dissociated by PMB and, as in the case of native enzyme, approximately 75% reconstitution to tetramer could be achieved by the addition of DTT. The effect of urea on reconstitution was not investigated. Finally, it was shown that the succinylated species formed hybrids with native GPDH upon dissociation by PMB and reconstitution by DTT (Figure 10).

To investigate the time dependence of dissociation of GPDH in the presence of PMB, we prepared mixtures of native (G_4) and succinylated (S_4) enzyme at pH 8 and at a concentration corresponding to that used in the previous experiments (approximately 8 mg/ml). PMB was added, and then at fixed times, DTT in excess was added. The resulting solutions were then left at 0–4° for several hours and examined by electrophoresis; typical results are shown in Figure 10. Clearly, when the enzyme has reacted with PMB for a considerable time (about 10 min) a hybrid set was observed indicating the formation of monomers as well as dimers. The hybrid S_3G is observed only in low amounts, either because it is thermodynamically less stable or because its rate of formation from the subunits upon addition of DTT is relatively slow. An identical result was obtained when the gel was stained for activity, although the relative band intensities were different as a result of the diminished activity of the succinylated enzyme component. That the DTT does in fact prevent formation of subunits by PMB is shown by the observation (Figure 10) that, when it is added immediately (within 5 sec) after the PMB, no hybrid bands are detected. It was found in several experiments (not shown here) that the material represented by the G_2S_2 band formed in considerable amount within 1 min of the addition of PMB. This band arises from hybridization at the dimer level and shows that dissociation of GPDH to dimers occurs within this time. The absence of the bands corresponding to G_3S and GS_3 shows that no monomers form within this time. The bands representing G_3S and GS_3 are seen to form at a much slower rate, judging from the slowly increasing intensity of the corresponding bands with time.

Effect of NAD on the Dissociation and Reactivation. Because an excess of the cofactor, NAD, has been shown to protect other dehydrogenases (Yonetani and Theorell, 1962; DiSabato and Kaplan, 1963; Dube *et al.*, 1963) and, in particular, lobster GPDH (Wassarman *et al.*, 1969), against inactivation by sulfhydryl reagents, the influence of NAD on the dissociation of the enzyme by PMB was investigated. Accordingly sedimentation velocity analyses of the type shown in Figures 1–3 were performed on solutions of GPDH containing NAD at a 100-fold excess (on a molar basis). The results at molar ratios of 4 and 12 for PMB:GPDH are indicated in Figure 3. As shown there the weight-average sedimentation coefficient or the fraction of enzyme dissociated was essentially the same whether NAD was present or absent. On inspection of the schlieren patterns, however, it was seen that the boundary representing the slower sedimenting material was somewhat sharper than that obtained in the absence of NAD. The equilibrium represented by the reaction boundary may have been shifted slightly, but not sufficiently to alter the values of $s_{20,w}$ significantly. In the presence of excess NAD the PMB-dissociated enzyme was less labile and did not precipitate on overnight standing at 0–4°.

Native GPDH preparations generally contain up to 4 moles of NAD/mole of tetramer, of which three are very firmly bound (Conway and Koshland, 1968; De Vijlder and Slater, 1968). Because the binding of NAD to GPDH is known to be weaker at pH 6.4 than at pH 8 (Cseke and Boross, 1967), it was thought that the pH dependence of the results described above might be in some way related to the altered affinity of the enzyme for its cofactor. Hence the dissociation and inactivation experiments were repeated with apoGPDH at pH 8. Samples of apoGPDH were reacted with various molar excesses of PMB, as before, and were assayed after suitable dilution both at zero time and after

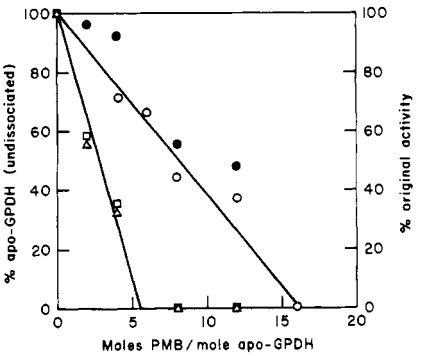

FIGURE 11: The effect of PMB on the quaternary structure and activity of apoGPDH. The symbol ○ represents the % dissociation of the apoenzyme as measured by the decrease in the weight-average sedimentation coefficient of the enzyme (5.1 g/l.) at pH 8, 7.7°; □ and △ represent the per cent activity remaining when assays were conducted at about 15 sec and 1 hr, respectively, after the addition of PMB at 7.7°. The samples (3.2 g/l.) were incubated in the Tris buffer (pH 8) and then diluted and assayed in the normal way. Following the assay at 1 hr, a 44-fold molar excess of NAD was added to the samples, which were then reassayed after a further hour of incubation at 7.7° (●).

1 hr. In a separate experiment the weight-average sedimentation coefficient was measured as a function of PMB and the per cent of undissociated enzyme was calculated. Both sets of results are presented in Figure 11. It is seen that for the apoenzyme total inactivation occurs upon reaction of approximately 5 moles of PMB/mole of enzyme and that no reactivation occurs upon standing for 1 hr (in the absence of NAD) as in the case of native GPDH. The amount of dissociation, however, was again proportional to PMB added with about 16 moles of PMB/mole of GPDH being required for complete dissociation.

To determine whether incubation with NAD leads to any reactivation of samples which had been reacted partially with PMB, we added a 44-fold molar excess of NAD to each of the samples; these were then assayed after 1-hr incubation at 7.7°. Although the results shown in Figure 11 are not as unequivocal as those obtained with native GPDH, it is clear that the addition of NAD leads to reactivation of apoGPDH which previously had been reacted partially with PMB. Presumably disproportionation occurs with PMB-treated apoGPDH at pH 8 but this process leads to the formation of inactive tetramers which require incubation with the cofactor, NAD, for reactivation.

Dissociation of GPDH by Other Mercurial Reagents. The enzyme was shown also to be reversibly dissociated at pH 8 by the mercurial 3-chloromercuri-2-methoxypropylurea (neohydrin), a compound which has the useful property that it does not absorb ultraviolet light of wavelength greater than 240 nm. GPDH which had been reacted with this substance was less labile than that reacted with PMB, a fact related presumably to the absence of the phenyl and charged carboxy groups in the former compound. The reactivity of neohydrin toward the enzyme is different from that of PMB, as indicated by the fact that excess NAD in the mixture gave partial protection againt dissociation by the former. The extent of dissociation of GPDH by neohydrin appeared to depend on the ratio of its concentration relative to that of NAD. With the increasing number of mercurials now available (McMurray and Trentham, 1969), compounds with other special properties may prove to be useful for the study of this enzyme.

Discussion

With the increasing realization of the role of subunit interactions in the functioning of multisubunit enzymes, specific methods of dissociating these proteins have become of importance. GPDH now joins a growing number of proteins known to be dissociated by PMB. These include such diverse types as aspartate transcarbamylase (Gerhart and Schachman, 1968), muscle phosphorylases *a* and *b* (Madsen and Cori, 1956), hemoglobin (Bucci *et al.*, 1965), pyruvate carboxylase (Palacian and Neet, 1970), succinyl-CoA synthetase (Grinnell and Nishimura, 1970), yeast alcohol dehydrogenase (Snodgrass *et al.*, 1960), hemerythrin (Keresztes-Nagy and Klotz, 1963), glutamate dehydrogenase (Rogers *et al.*, 1963), glycyl-tRNA synthetase (Ostrem and Berg, 1970), and formyltetrahydrofolate synthesase (Nowak and Himes, 1971). Yet in most cases the mechanism of the dissociation and its relationship to inactivation of the enzymes have not been elucidated.

From the results obtained in the present work, it is concluded that the enzyme GPDH contains three classes of SH group, each with differing reactivity toward PMB. This conclusion is made because, although the PMB reaction kinetics indicated two *major* classes of groups, total enzyme inhibition was initially observed after reaction of four groups per molecule. Therefore, of the fourteen[2] reacting SH groups on the enzyme, there are seven slowly reacting groups and seven fast-reacting groups, the latter being themselves heterogeneous in their reactivity toward PMB. Because GPDH consists of four identical subunits (Harris and Perham, 1968), it is likely that enzymic inactivation corresponds to the reaction of one SH group per chain;[3] this inactivation is independent of pH to the extent that it occurs at both pH 6.4 and 8. These results therefore confirm the kinetic results of Vas and Boross (1970) who also demonstrated a triphasic mechanism for the reaction of GPDH with PMB.

During the reaction of the first seven SH groups, at pH 8, the protein dissociates into dimers, since the time in which they react corresponds well with the time for maximal production of hybrid (G_2S_2) as seen in Figure 10. No electrophoretic bands indicative of monomer hybrids were observed in this time, however. Whether the dissociation occurs upon reaction of those four groups whose reaction leads to inactivation of the enzyme has not been determined. During the reaction of the seven slow groups the enzyme is apparently subjected to a further, slower, conformational rearrangement, facilitating the formation of monomers, as observed by the slow formation of the G_3S (and a slight amount of GS_3) hybrid. It appears, therefore, that the reaction of GPDH with PMB causes structural changes in the protein in two major stages. The first stage, in which the enzyme is dissociated to dimers, accompanies reaction of the rapidly reacting classes of SH groups. The second stage, accompanying reaction of the slow SH groups, brings about a further structural change in the molecule which allows dissociation to monomeric subunits. The hybridization technique, of course, gives no indication of the relative values of the equilibrium constants and in fact sedimentation studies showed that, after fully reacting with PMB, the enzyme exists predominantly as a mixture of dimers and tetramers in rapid equilibrium. Presumably this second stage of the reaction is the one observed spectrally by Friedrich and Szabolcsi (1967). They showed that in swine GPDH the rate constant for the ultraviolet spectral change observed upon reaction with PMB was equal to the pseudo-first-order rate constant for the slowly reacting class of SH groups in that enzyme. Of considerable interest would be a comparative study of the action of PMB on GPDH enzymes isolated from mammalian, lobster, and yeast tissues. These enzymes contain four, five, and two SH groups per subunit, respectively, and are homologous in only two of these groups (Harris and Perham, 1968). One might then be able to determine whether one specific group in each subunit is responsible for dissociation of the enzyme. At pH 6.4, the situation is different, since virtually no dissociation is observed until more than seven PMB molecules are added per molecule of GPDH (Figure 5). In this case, therefore, dissociation only accompanies the reaction of the slowly reacting class of SH groups.

When less than 14 moles of PMB was added per mole of enzyme, an all-or-none dissociation of the enzyme was observed after a certain time. Elödi (1960) has shown that when swine muscle GPDH is reacted with PMB at pH 8.4, both the intrinsic viscosity and optical rotation of the solution increase linearly with PMB concentration until 14 moles of PMB/mole of enzyme have been added. In view of the close similarities between the various mammalian enzymes (Harris and Perham, 1968) it seems likely that these parameters also reflect on all-or-none *dissociation* of the molecule.

The observation of the all-or-none nature of the dissociation in partially reacted mixtures at pH 8 is explained in terms of a disproportionation of PMB groups following their initial relatively uniform reaction with all of the protein molecules (Szabolcsi *et al.*, 1960). Four sets of data, in conjunction with the ultracentrifuge experiments showing all-or-none reaction with PMB, support this conclusion. First, the kinetics of mercaptide-bond formation indicate at least two major classes of SH reactivity toward PMB, suggesting that all GPDH molecules react partially with PMB before any react to completion. Second, total inactivation occurs very rapidly upon the addition of only four PMB molecules per molecule of enzyme. Third, changing the pH, from 6.4 to 8, of a sample which had been reacted with 7 moles of PMB/mole causes the enzyme to be converted from an essentially homogeneously sedimenting species into a mixture of dissociated, fully reacted, species and native tetramers. This result, together with the demonstration that at pH 6.4 the SH groups are again heterogeneous with respect to PMB reactivity, strongly indicates that the disproportionation can be induced merely by altering the pH of the solution. Finally, samples of GPDH which had been partially reacted with PMB at pH 8 and were devoid of activity were found to regain activity upon standing. This result was first demonstrated by Szabolcsi *et al.* (1960) and the present work extends their findings in demonstrating that the final per cent of original activity in the solution is proportional to the per cent of native tetramer in the mixture, irrespective of the amount of PMB added.

[2] Both the dissociation results and the kinetic results indicated that a total of 14 groups on the molecule react. Szabolcsi *et al.* (1960) also obtained this number for the pig enzyme. In view of the demonstration of 16 cysteinyl residues in these enzymes (Harris and Perham, 1968), the reason for this number is unclear. It is possible that the native enzyme when isolated is actually acylated at two SH groups, thereby precluding reaction of PMB at these sites (Bloch *et al.*, 1971). Alternatively the masking of two SH groups may in some way be related to NAD binding since for the apoenzyme 16 PMB groups/mole of enzyme appeared to be required for full dissociation.

[3] Wassarman *et al.* (1969) attributed the inactivation by PMB of lobster GPDH to reaction of cysteinyl residue 152, which suggests that the corresponding residue number 153 in the mammalian enzyme (Harris and Perham, 1968) is the first to react with PMB. This group is very close to, but apparently not part of, the active site SH group which reacts with iodoacetic acid (Wassarman *et al.*, 1969). It is of interest that we have observed that an excess of iodoacetate of pH 8 results in no dissociation of the enzyme within 4 hr.

Of particular interest is the nature of the driving force for the disproportionation. Presumably the order in which the SH groups react is dictated by kinetic considerations, whereas the thermodynamic requirements of the system dictate the composition of the final solution in terms of equally reacted molecules or a mixture of native, intact molecules and fully reacted, dissociated species. The hybridization data show that this dissociation to dimeric subunits is a fast process, suggesting that the disproportionation occurs at the subunit level. If this is the case, the disproportionation must be followed by a reassociation to native molecules. Apparently it is the decrease in free energy resulting from as many as possible of the protein molecules acquiring their native, tetrameric, conformation which provides the driving force for the disproportionation, a concept which has interesting ramifications toward an understanding of the pathways by which protein chains fold and aggregate to oligomers. Alternatively disproportionation may be attributed to a lowering of free energy stemming from some of the partially reacted (and presumably partially dissociated and unfolded) molecules acquiring a completely denatured state. In this regard it is interesting to note that Szabolcsi *et al.* (1960) have shown that GPDH molecules which were apparently irreversibly denatured by heat treatment facilitate reactivation of GPDH which had been inactivated by total reaction with PMB (15 moles/mole of enzyme). Although not demonstrated conclusively it appears that the potentially reactivatible mercurial-treated molecules donate their mercurials to the heat-denatured polypeptide chains so that the former can acquire the structure of the native enzyme. A demonstration of the transfer of mercurials from one type of chain to the other would provide strong evidence that the former explanation of the driving force for the disproportionation is the correct one. It is possible that the SH groups with the highest binding constant for PMB are those which react most slowly for steric reasons and that after dissociation (and presumably unfolding) of the protein these groups with the high affinity become available. Disproportionation would then be viewed as the migration of mercurials from low-affinity to high-affinity SH groups. Such a possibility cannot be ruled out although as yet widespread differences in the affinity of SH groups of protein for mercurials have not been demonstrated. The differences in behavior of GPDH at pH 8 and 6.4, despite similarities in the kinetics of mercaptide formation, suggest that disproportionation occurs in the former case and not in the latter because of variations in the conformation of the enzyme (and the mercurial derivative) at the two pH values and not because of major differences in the affinity of SH groups for PMB. The results with apo-GPDH, although not complete, suggest that NAD is required, not for the formation of tetramers following disproportionation, but for the formation of enzymically active species from inactive tetramers.

It is of interest that both aspartate transcarbamylase (Gerhart and Schachman, 1968) and phosphorylase (Madsen and Gurd, 1956) dissociate in an all-or-none manner with respect to PMB. It seems unlikely, however, that a disproportionation mechanism pertains to aspartate transcarbamylase since the kinetics of mercaptide formation indicated a single rate constant, suggesting that the reaction of one or a few of the SH groups was rate limiting. Some type of "zipper" mechanism therefore seems most likely for this enzyme. A disproportionation mechanism cannot be fully eliminated, however, in the case of phosphorylase *b*, which has been shown to possess thiol groups of differing reactivity toward a number of reagents (Gold, 1968). Of course, the nature of the dissociated subunits would be expected to vary from enzyme to enzyme. For example, whereas GPDH is dissociated into subunits which are in equilibrium with the tetrameric form of the enzyme, the subunits produced from aspartate transcarbamylase and phosphorylase apparently are not. Disproportionation has been implied, without proof, by Robinson *et al.* (1967) to explain the time-dependent reactivation of fumarase following initial inactivation with PMB. Regeneration of activity has also been observed with the enzymes xanthine oxidase (Westerfeld *et al.*, 1959), leucine decarboxylase (Sutton and King, 1962), and lactate dehydrogenase (Gruber *et al.*, 1962), all of which may resemble GPDH in their behavior toward PMB. This reactivation phenomenon has also been observed with other mercury compounds (Webb, 1966) and in GPDH with Ellman's reagent (Boross, 1969). It could be responsible for anomalies existing in the literature in connection with kinetic studies relating enzymic inhibition to inhibitor concentration. For example, Listowsky *et al.* (1965) reported that reaction of 4 moles of PMB/mole of rabbit muscle GPDH reduced enzymic activity to 49% of the original activity; in the absence of information about the activity as a function of time between PMB addition and enzymic assay, it is not clear whether the molecules were first inactivated and then partially reactivated. Disproportionation may be of interest in studies of other biological molecules. For example, Hong and Rabinowitz (1970a,b) have shown that iron and sulfide bind to, and can be displaced from, ferredoxin in an all-or-none fashion. They showed that iron and sulfide undergo base-catalyzed exchange reactions, which could presumably provide the basis for a disproportionation mechanism resulting in the observed all-or-none binding. In addition α,α'-bipyridyl and mersalyl reacted in an all-or-none manner.

The observation that fully reacted GPDH is predominantly converted to the dimeric form is further evidence for the isologous nature of the intersubunit bonding domains (Meighen and Schachman, 1970a). Since monomers were detected by the hybridization studies, the approximation which the system shows to a dimer–tetramer equilibrium indicates that the monomer–dimer association constant is very high. Apparently then, an effect of PMB on the enzyme is to lower greatly its dimer–tetramer association constant.

It is of interest that a relatively homogeneous derivative has been achieved after reaction of GPDH with succinic anhydride, by a modification of the procedure of Meighen and Schachman (1970b). It was seen that succinylation at cold temperature in the presence of an excess of NAD prevents formation of a dissociated product of the enzyme even upon maximal reaction as judged by electrophoretic mobility. The reason for this protection is unknown, since the native enzyme itself contains three to four firmly bound NAD molecules per GPDH molecule. NAD, when added in excess, is known to protect the enzyme against spontaneous inactivation (Velick and Furfine, 1963) perhaps by binding to the protein and, thereby, helping to maintain a stable conformation. The availability of a homogeneous, charged variant, which is capable of hybridizing with the native protein, should prove useful in further studies of the nature of the subunit interactions in the molecule and for investigating other conditions of dissociation of the enzyme. In particular, the hybridization technique provides a novel approach to the determination of approximate rates of dissociation of oligomeric proteins.

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