Glucose 6-Phosphate Dehydrogenase from Brewers’ Yeast
(Zwischenferment)

III. STUDIES ON THE SUBUNIT STRUCTURE AND ON THE MOLECULAR ASSOCIATION PHENOMENON INDUCED BY TRIPHOSPHOPYRIDINE NUCLEOTIDE

(Received for publication, November 1, 1968)

ROBERT H. YUE, ERNST A. NOLTSMANN,‡ AND STEPHEN A. KUBY
From the Laboratory for the Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah, Salt Lake City, Utah 84112

SUMMARY

Sedimentation equilibrium studies of the glucose 6-phosphate dehydrogenase apoprotein (mol wt 101,600 g per mole), conducted in the presence of guanidinium chloride, permit the conclusion that it is composed of two polypeptide chains of 51,000 molecular weight with similar, if not identical, physical properties. Elemental metal analysis on the apoprotein for nine different metals has failed to reveal significant concentrations of a metal constituent. Contrary to previous reports, this enzyme is not a zinc metalloprotein.

The observations of Noltmann and Kuby (in P. D. BOYER, H. LARDY, AND K. MYRNÄCK (Editors), The enzymes, Vol. 7, Academic Press, New York, 1963, p. 223) on the triphosphopyridine nucleotide-induced association phenomenon of the protein have been confirmed by sedimentation equilibrium measurements conducted in the presence of excess TPN⁺. The TPN-enzyme compound is the “dimer” of the apoprotein; i.e. it consists of four subunits. The apoenzyme may be titrated with TPN⁺ to alter successively its hydrodynamic properties, and sedimentation velocity studies in the presence of appropriate concentrations of TPN⁺ reveal this TPN-enzyme, with a sedimentation coefficient larger than that of the apoenzyme. Values for its $s_{20,w}^0$ and $D_{20,w}^0$ have been deduced, and a prolate model for the tetra-chain species is proposed.

Ethylendiaminetetraacetate, if present at significant concentrations, will completely inhibit the TPN-induced association reaction. This has been interpreted as an effect of the EDTA⁺ ion on solvent-macromolecular interactions, modifying the dissociation-association equilibria, rather than an effect due to its chelation properties. In the absence of interacting ionic species, successful ultracentrifugal titrations have been conducted and interpreted quantitatively in terms of a set of equilibria involving the successive binding of TPN⁺ to its subunits. The controlled use of EDTA will permit a simplified approach to studies on the catalytic mechanism (mechanisms) through direct steady state kinetic measurements on the active two-chain species.

In the first paper of this series (1; cf. also Reference 2) the isolation from brewers’ yeast of n-glucose 6-phosphate dehydrogenase (Zwischenferment) as the crystalline TPN-compound was described. The second paper (3) dealt with the homogeneity of this protein when essentially freed of its coenzyme, TPN⁺ (i.e. the TPN-free species or the apoenzyme). This apoprotein could also be obtained in crystalline form which, interestingly, is geometrically different from that of the TPN-compound. Physical measurements on the isolated apoprotein (3) yielded an assigned value for its kinetic molecular unit of 102,000 g per mole. The present report includes ultracentrifugation studies conducted in the presence of guanidinium chloride, which led to the conclusion that the 102,000-g unit of the apoprotein is composed of two polypeptide chains. The identity or nonidentity of these two chains will have to be established by chemical analysis but, tentatively, the physical data point to identical chains; i.e. it appears likely that the apoprotein molecule is actually a two-chain dimer. These conclusions concerning the yeast enzyme contrast with those drawn by Yoshida (4) for human erythrocyte glucose 6-phosphate dehydrogenase which he believed to have three chains; however, there may have been some uncertainty in the corrections used in that case for guanidinium binding. Moreover, Yoshida’s interpretation (4) is at variance with the recent results of Rattazzi (5) who reached analogous conclusions (by gel filtration studies) regarding the human erythrocyte enzyme as deduced here for brewers’ yeast glucose 6-phosphate dehydrogenase.

Vallee et al. (6) had very early conducted an elemental metal analysis on the enzyme from yeast (a commercial preparation).
and had found significant amounts of lead, iron, zinc, copper, calcium, and magnesium. Since the specific activity of that preparation (6) was very likely not higher than a few per cent of the crystalline enzyme (1), these early results lack significance; however, Vallee et al. (6) had also focused attention on zinc as a possible metal constituent of the yeast enzyme on the basis of inhibition studies with metal chelators. Furthermore, Nevaldine and Levy (7) from studies on the mammary gland glucose 6-phosphate dehydrogenase appeared to confirm the inhibition studies of Vallee et al. It is shown here, however, that quantitative metal analyses on the apoenzyme for nine different metals failed to reveal the presence of significant concentrations of any of these in the protein preparation. It does not appear likely, therefore, that yeast glucose 6-phosphate dehydrogenase is a "metal" enzyme in the classical sense, or that zinc, at least, is a metal constituent of the yeast enzyme. The classical chelating agent EDTA, however, does exert a profound influence on the molecular association phenomenon induced by TPN++; yet, the yeast enzyme is catalytically active in the presence of EDTA.

Finally, this report is concerned with some observations and attempts to characterize the TPN-compound and with studies that bear on the interesting molecular association phenomenon induced by the presence of its coenzyme, TPN. Noltmann and Kuby, in a review (8), quoted unpublished observations (which are presented here) clearly indicating that the presence of TPN\textsuperscript{1} dramatically alters the hydrodynamic properties of the enzyme protein. Specifically, they had found that the ultracentrifugation behavior of the protein was significantly affected by the presence of TPN\textsuperscript{+}. The addition of TPN\textsuperscript{+} to the apoenzyme resulted in a considerable increase in the sedimentation velocity. By choosing the appropriate TPN concentrations, the change from the apoenzyme into another species with a larger sedimentation coefficient could be demonstrated, and it was actually possible to show a separation of the TPN-enzyme compound and of the free enzyme in the ultracentrifuge cell. The molecular weight of the TPN-enzyme compound was calculated by Noltmann and Kuby (8) from the sedimentation coefficients and with estimates of the diffusion coefficients from the boundary spreading during sedimentation; the TPN enzyme appeared to have twice the molecular weight of the apoenzyme. These early observations have now been confirmed by a more thermodynamic means, i.e., sedimentation equilibrium experiments conducted in the presence of a large excess of TPN\textsuperscript{+}. It is thus apparent that the two-chain apoenzyme can further dimerize to yield the final TPN-compound, consisting of two apoenzyme half-molecules each composed of two subunits.

In the course of the more recent studies, it was found that EDTA is a potent inhibitor of the TPN-induced molecular association process. The mechanism by which EDTA exerts its effect does not involve its chelation properties; rather, specific charge interactions, as related to solvent-macromolecular interactions, may play a role in modifying monomer-polymer equilibria (10). With this information as a clue, the design of initial kinetic studies was greatly simplified by having a means to render the association process negligible. Such studies on the catalytically active "monomer,"\textsuperscript{2} which predominates in the presence of EDTA, have now been successfully carried out and will be the subject of a future communication. Present efforts are directed toward a study of conditions which will permit a clearer understanding of the effect of this interesting "dimerization" reaction (or reactions) on the catalysis and on the kinetic mechanism. Ultimately, it is hoped that a kinetic model will become available for elucidating quantitative aspects of the current concepts of allosterism (11, 12) and of the induced fit hypothesis (13-15). Further studies on the interactions of the enzyme with its several substrates are being approached by several techniques by which it is hoped also to shed further light on the mechanism of action of this dehydrogenase and, by extrapolation, on the means whereby it might exercise a role in the control of carbohydrate metabolism.

**EXPERIMENTAL PROCEDURE**

**Materials**

_Enzyme Preparations—Glucose 6-phosphate dehydrogenase (Preparations 11 to 14), used for the earlier studies mentioned in the review by Noltmann and Kuby (8), had been isolated from brewers' yeast by the procedure originally described (1). For the later studies the enzyme was isolated by the slightly modified procedure (2) adapted for large scale preparation (Preparations 19 to 23). After four to six crystallizations of the enzyme from aqueous (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} solutions containing TPN (1), the enzyme reached constant specific activity. These preparations of the crystalline TPN-enzyme were freed of TPN++ as described previously (3) and the apoenzyme was then crystallized. The dissolved apoenzyme crystals were dialyzed against 0.01 M EDTA-0.05 M phosphate (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}-2.145 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} pH 6.8, and stored at -10 to -15°. Aliquots of the apoprotein solution were dialyzed exhaustively against the appropriate aqueous solvent or buffer used in each of the measurements, except in experiments with guanidinium chloride for which they were treated as described below._

_Other Materials_ TPN++ (free of EDTA) was obtained from Sigma. Other buffers and chemicals were the best available analytical grade commercial products. The various metal standards were prepared from suitable salts, dissolved in twice distilled deionized water or in HCl solutions (diluted from redistilled constant boiling HCl), and stored in polyethylene containers; some metal samples were obtained from the National Bureau of Standards. Solutions for the sedimentation measurements were prepared as described in Reference 3. When necessary, dissolved traces of CO\textsubscript{2} in the redistilled deionized water were removed by boiling.

**Methods**

_Sedimentation Velocities—These were measured at 3° (or 3-5° for the earlier studies) in a Spinco model E analytical ultracentrifuge._

\textsuperscript{2} For simplicity, in this paper the reaction or reactions of TPN++ with the apoprotein of two subunits (each with mol wt 51,000) to yield the TPN-enzyme of four subunits is termed "dimerization," although, strictly speaking, this would represent a tetramerization. The term "monomer" (P) thus refers to the two-subunit apoprotein of 101,600 g per mole and "dimer" (P\textsubscript{2}) to the tetramer- subunit TPN-enzyme of twice the molecular weight of the apoprotein. Work is currently in progress to establish the chemical identity or nonidentity of the 51,000 molecular weight subunits.

\textsuperscript{1} R. N. Roy and S. A. Kuby, unpublished experiments; manuscript in preparation.

\textsuperscript{2} A first report on the TPN-induced, molecular association of glucose 6-phosphate dehydrogenase was given in April 1961 at the 45th Meeting of the American Society of Biological Chemists in Atlantic City. Additional data were presented in 1966 at the 50th Meeting of the American Society of Biological Chemists in Atlantic City (cf. also Reference 9).
trifuge equipped with an RTIC temperature control system and a phase plate as a schlieren diaphragm (the schlieren optical system was aligned according to Gropper (16)). Kodak metallographic plates were used to photograph the schlieren patterns with a Kodak Wratten 77A filter over the light source. Most of the work was performed with a single sector cell of 12-mm optical path. Sedimentation coefficients obtained at 3° and in buffer (s20,w) were calculated in the usual fashion (17) and were converted to s20,∞ values (18) with the use of density and viscosity data measured for the buffers and the solvents used and with a value for the partial specific volume of the apoprotein (3).

For the ultracentrifugal titrations of the apoprotein with TPN+, aliquots of TPN+ in 0.15 M NaCl, pH 6.8, were successively added to a protein solution of approximately 6 mg per ml of initial concentration, prior to the sedimentation velocity measurements. Progressive splitting of the boundary into two components was evident with increasing concentrations of total TPN+ (TPN), and was accompanied by a qualitative increase in the faster sedimenting component. The photographed patterns were enlarged (Omega type DII) and traced onto graph paper, and the areas beneath the graphically separated boundaries were determined planimetrically. For the older work, which is reported here, runs had been conducted in 0.01 M KCl, with varying amounts of TPN+ added to protein solutions of 5 to 10 mg per ml; the boundary areas had been estimated by application of Simpson's rule.

The respective areas for heavy and light component were corrected for radial dilution during the run and in turn corrected for the Johnston-Ogston effect (19, 20), i.e.

\[
\frac{(X_x^{20s} / X_m)^n}{C_x^{obs}} = \frac{(X_x^{20s} / X_m)^{(n-1)} - 1}{(X_x^{20s} / X_m)^n - 1}
\]

and

\[
\psi = \frac{S_x^2}{S_p^2}
\]

The nomenclature follows that of Schachman (19) where Cx^{obs} and C_x are, respectively, the observed concentration of the slow component and the slow component concentration corrected to zero time (expressed ultimately in milligrams per ml); i.e. C_x corresponds to the value before separation by ultracentrifugation; X refers to the distance (corrected for magnification, etc.) from the axis of rotation; subscript m, to the meniscus position; f_x to the fast component, and s, to the slow component. \(X_x\) or \(X_f\) should correspond to the distance of the slow or the fast component boundary from the axis of rotation, as measured from the square root of the second moment of the gradient curve; for fairly symmetrical boundaries, these positions could be approximated by the positions of the maximum ordinate. To obtain \(\psi\) from \(S_x^2\) and \(S_p^2\) (the sedimentation coefficients of the slow and fast components at infinite dilution), the sedimentation coefficients of the individual components (under conditions of the titrations conducted in 0.01 M phosphate-0.15 M KCl (pH 6.8)) were estimated from eight runs of the older data. As a whole, these data provided a set of widely varying concentrations of fast (and slow) component for which the total protein concentration (P1) ranged from approximately 5 to 10 mg per ml. These values were corrected to the reference states of 20° and water and were extrapolated to infinite dilution to yield values of \(S_{20,∞}\) for the fast and slow components and, therefore, a value of 0.657 for \(\psi\). For justification of this value for \(\psi\), see “Results.”

**Sedimentation Equilibrium**—These experiments were performed at 3° for molecular weight measurements of the TPN-protein (the apoprotein to which a large excess of TPN+ had been added; details can be found in the legends to the appropriate figures), and at 20° for the subunit analyses of the apoprotein in guanidinium chloride. These runs were made with the use of a rotatable light source and Rayleigh interferometric optics (21) which were aligned according to Gropper (16). A double sector synthetic boundary cell of the capillary type with sapphire windows and 12-mm optical path was used, and Kodak II G plates served to record the interferograms. Details as to the techniques, procedures, and calculations used for the low speed and the meniscus depletion high speed (22) sedimentation equilibrium analysis have been reported in previous communications (3, 23).

Two procedures for preparation of samples for the physical measurements in guanidinium chloride or urea were followed: Procedure “a” was that previously used for rabbit muscle ATP-creatine transphosphorylase (23), viz. exhaustive dialysis against redistilled water, followed by lyophilization of aliquots of salt-free protein in vacuo, and finally dissolution in the selected solvent. For these determinations, the following solvents were used: 8 M urea or 4 M guanidinium chloride containing either 0.001 M EDTA or 5 × 10^-4 M 1,10-phenanthroline and 0.1 M 2-mercaptoethanol, at pH values ranging from 2 to 6.1. Because the apoprotein tended to crystallize during the dialysis at low ionic strengths (3), Procedure “a” for desalting sometimes resulted in formation of protein crystals which had to be redissolved, sometimes with difficulty, in the denaturing solvents. The second procedure, “b,” obviated this difficulty by equilibrating (by dialysis) the protein solutions in 0.01 M Tris (pH 7.8), followed by lyophilization and redissolution in the solvent used for chain disruption (4 M guanidinium chloride-0.001 M EDTA-0.1 M 2-mercaptoethanol, pH 6.3): under these conditions rapid and complete dissolution occurred. Calculations of the molecular weight averages were performed as described previously (23).

**Elemental Metal Analyses**—These were conducted by atomic absorption. Samples of the apoprotein solution were exhaustively dialyzed against 0.05 M KCl-0.005 M Tris (pH 7.5), 8 M urea. These samples (approximately 0.4 mg per ml) were subjected to metal analysis by means of a recording Perkin Elmer model 303 atomic absorption spectrophotometer equipped with read-out scale expansion accessory, air-acetylene “pre-mix,” and wide slit 10-cm burner head; samples were aspirated into the burner at a rate of 4 ml per min per siphon for at least 30 sec per analysis. Solutions of the dialysis fluid were analyzed in a similar manner. To ensure that the water solutions of protein did not present any interference to the instrumental analysis, additional samples (5 mg) were dry-ashed in platinum boats. The ash was dissolved in 0.1 N HCl (prepared by dilution from redistilled constant-boiling HCl, with double distilled deionized water) and subjected to the same analyses. Hollow cathode lamps containing either single elements or multielement sources were used for zinc, calcium, copper, iron, magnesium, manganese, molybdenum, cobalt, and chromium. (We are indebted to R. J. Heaney of Kennecott Copper Research Center for making available to us the lamp for manganese, cobalt, and chromium and to Professor J. E. Wilson of the Department of Mining and Geological Engineering for providing a number of lamps.) Standard solutions of each element were measured over a range of con-
The apoprotein was dissolved in urea or guanidinium chloride solutions at several pH values and under several sets of conditions at 20°. NaAc, sodium acetate; RSH, 2-mercaptoethanol; OP, o-phenanthroline.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Procedure</th>
<th>$M_w$</th>
<th>Time at equilibrium speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20 mg/ml, 8 m urea, 0.05 m NaCl, 0.05 m NaAc, 0.001 m EDTA, 0.01 m RSH, pH 5.100</td>
<td>a</td>
<td>63,400</td>
<td>30</td>
</tr>
<tr>
<td>0.20 mg/ml, 8 m urea, 0.05 m NaCl, 0.05 m NaAc, 0.001 m EDTA, 0.01 m RSH, pH 5.100</td>
<td>a</td>
<td>62,700</td>
<td>28</td>
</tr>
<tr>
<td>0.20 mg/ml, 4 m guanidinium chloride, 0.05 m NaAc, 0.001 m EDTA, 0.01 m RSH, pH 5.100</td>
<td>a</td>
<td>61,700</td>
<td>32</td>
</tr>
<tr>
<td>0.20 mg/ml, 4 m guanidinium chloride, 0.05 m Na formate, 0.005 m OP, 0.1 m RSH, pH 3.500</td>
<td>a</td>
<td>55,900</td>
<td>30</td>
</tr>
<tr>
<td>0.20 mg/ml, 6 m guanidinium chloride, 0.05 m Na formate, 0.005 m OP, 0.1 m RSH, pH 3.500</td>
<td>a</td>
<td>58,000</td>
<td>30</td>
</tr>
<tr>
<td>0.20 mg/ml, 4 m guanidinium chloride, 0.05 m Na formate, 0.005 m OP, 0.1 m RSH, pH 3.500</td>
<td>a</td>
<td>59,500</td>
<td>30</td>
</tr>
<tr>
<td>0.20 mg/ml, 4 m guanidinium chloride, 0.005 m OP, 0.1 m RSH, pH 2.000</td>
<td>a</td>
<td>59,800</td>
<td>30</td>
</tr>
<tr>
<td>0.20 mg/ml, 4 m guanidinium chloride, 0.05 m glycine, 0.005 m OP, 0.1 m RSH, pH 2.000</td>
<td>a</td>
<td>57,600</td>
<td>30</td>
</tr>
<tr>
<td>0.25 mg/ml, 4 m guanidinium chloride, 0.001 m EDTA, 0.10 m RSH, pH 6.300</td>
<td>b</td>
<td>51,100</td>
<td>28</td>
</tr>
<tr>
<td>0.25 mg/ml, 4 m guanidinium chloride, 0.001 m EDTA, 0.10 m RSH, pH 6.300</td>
<td>b</td>
<td>51,000</td>
<td>28</td>
</tr>
<tr>
<td>0.25 mg/ml, 4 m guanidinium chloride, 0.001 m EDTA, pH 6.300</td>
<td>b</td>
<td>60,400</td>
<td>28</td>
</tr>
</tbody>
</table>

* See "Methods."

concentrations, at suitable wave lengths, to establish the sensitivity and precision of the analyses, and standards were run with each set of analyses.

RESULTS

Sedimentation Equilibrium Studies on the Polypeptide Chains of the Apoprotein in Denaturing Agents—A large number of runs were conducted by Yphantis' procedure (22) in the presence of urea or guanidinium chloride to find conditions which would yield a maximum extent of dissociation. These runs are summarized in Table I in terms of the weight average molecular weight throughout the cell contents for each of the several conditions explored. For most of these runs, Procedure "a" (see "Methods") was used. At first, it appeared that in the presence of 8 m urea, at ionic strengths near the apparent isoelectric point of the native molecule (3), with or without 2-mercaptoethanol (but in the presence of 10−3 m EDTA), incomplete dissociation resulted. Guanidinium chloride (4 to 6 m) was subsequently selected as the most powerful of the disruptive agents which had previously and successfully been applied to rabbit muscle ATP-creatine transphosphorylase (23). Under the mistaken assumption, at the time, that the apoprotein might contain zinc (6, 7) and that it may follow the association-dissociation behavior of zinc-insulin (e.g. Reference 244 and references quoted therein), measurements were then conducted in 4 m guanidinium chloride containing 5 × 10−4 m o-phenanthroline and 0.1 m 2-mercaptoethanol over a range of several pH values. Moreover, from the electrophoretic data, $p_i = 6.0$ (3), the apparent isoelectric point would be expected to be shifted at high ionic strength to a value far below 5.0, and charge effects and preferential binding of guanidinium ion should be minimized at the apparent isoelectric point of the protein. In terms of the apparent $M_w$ value as a function of pH, a minimum in $M_w$ (approximately 55,900) was actually seen at about pH 3.5 (20°) for 4 m guanidinium chloride; this value may be compared with a hypothetical value of 51,300 for a half-molecule (3). Increasing the guanidinium chloride concentration to 6 m did not decrease the value for $M_w$ further. Under all of these conditions it was difficult to decide whether real evidence for nonideality or aggregate formation could be deduced from the Yphantis plots.

Some difficulties were also encountered in producing complete dissolution of the crystalline protein in the solvents used for chain disruption. With the use of Procedure "b" (see "Methods"), and by substituting EDTA as a chelating agent to bind trace metals still remaining in the guanidinium chloride (which had been purified by the procedure of Nozaki and Tanford (25)), and in the presence of 0.1 m 2-mercaptoethanol (redissolved under reduced pressure), successful and quantitative disruption of the chains resulted in 4 m guanidinium chloride, pH 6.3 (Fig. 1A; Table I). A weight average value of 51,000 is obtained with little, if no significant, differences between the molecular weight averages calculated for the entire cell content (i.e. $M_w \approx M_o \approx M_z$).

* See especially page 188 ff.
In the absence of 2-mercaptoethanol, however, a small rise was observed in the weight average molecular weight throughout the cell (Fig. 1B); this is probably the result of a slight oxidation with interchain disulfide formation and concomitant aggregation which is noticeable at the bottom of the cell. It is, however, apparent that the kinetic unit apoprotein molecule of 101,600 molecular weight (3) consists of two polypeptide chains with no interdisulfide bonds linking the chains. Within experimental error, both chains appear to be physically identical, but the final proof of chemical identity of these chains must await analyses of the terminal groups and tryptic peptides. This proposed model of a two-chain molecule for the apoprotein contrasts with Yoshida's conclusion (4) that the erythrocyte enzyme molecule consists of three chains; it appears, however, that there may be some uncertainty in his estimated molecular weight of the native TPN-free molecule, as well as in the corrections which Yoshida had used (4) for guanidinium binding and for the effect of the guanidinium chloride on the partial specific volume of the erythrocyte enzyme. On the other hand, Rattazzi's results (5) on the erythrocyte enzyme molecule appear to be in good agreement with those given here for the yeast protein.

**Elemental Metal Analysis of the Apoprotein**—These data are summarized in Table II. Atomic absorption analyses conducted at approximately 0.4 mg per ml of protein did not show any of the analyzed metals to be present at mole ratios exceeding approximately 0.2 g atom/101,600 g of protein. These metal analyses, which included zinc, calcium, copper, iron, magnesium, manganese, molybdenum, cobalt, and chromium may be taken as indicative of negative results, especially for zinc, which proved to be present below the detection limit of 0.02 g atom per mole.

During tests of the sensitivity and precision of the method used, bovine serum albumin at approximately 0.5 mg per ml proved to be essentially free of zinc and the addition of zinc (0.519 µg per ml) resulted in 103% recovery. Interestingly, however, traces of copper at approximately 0.070 g atom per mole of protein could be detected in bovine serum albumin. This should be kept in mind when the albumin is used as a "protective agent" for enzyme assays since, at a concentration of 1 mg per ml, approximately 10⁻⁴ M copper would be introduced into the reaction mixture. Finally, analyses of a commercial sample of carboxypeptidase A gave about 0.9 g atom of zinc per mole of protein under conditions for which the precision of the method was 3%, compared to a theoretical value of 1.0 (26). Consequently, had stoichiometric concentrations of zinc been present in glucose 6-phosphate dehydrogenase, this technique (whose validity, sensitivity, and accuracy had been found adequate as evidenced by the control runs) would have certainly revealed its presence. The earlier results of Vallee et al. (6) on a commercial preparation of glucose 6-phosphate dehydrogenase of doubtful purity now seem to lack significance. The yeast enzyme does not contain zinc, or any of the other eight metals for which analyses had been conducted.

Finally, the enzyme inhibition studies of Vallee et al. (6) with metal chelators on the yeast preparation and possibly those studies of Nevaldine and Levy (7) on rat mammary gland glucose 6-phosphate dehydrogenase may be reinterpreted as possibly due to catalytic oxidation of thiol groups, perhaps by the mechanism shown by Kobashi (27) and Kobashi and Horecker (28) for rabbit muscle aldolase with the use of an o-phenanthroline-copper complex. The danger of utilizing inhibition by so-called specific metal chelators as the sole means to prove or disprove the presence of a metal cofactor in an enzyme preparation seems evident from these results. For the present, therefore, the postulate that the yeast glucose 6-phosphate dehydrogenase contains a tightly bound metal constituent can no longer be considered as tenable.

**Studies on the TPN-enzyme**—Noltmann and Kuby (8) had first noted that the hydrodynamic behavior of the apoprotein was dramatically affected by the presence of its coenzyme, TPN⁺. In Fig. 2, some typical patterns are presented from the earlier sedimentation velocity runs which had been made at three different initial TPN⁺ concentrations added to 0.15 M KCl-0.01 M phosphate (K⁺), pH 6.8 at 3⁰. The addition of TPN⁺ to the apoprotein, even in the presence of dilute phosphate buffer, clearly resulted in a considerable increase in the sedimentation velocity. By choosing the appropriate TPN⁺ concentration added to a given total protein concentration, the change from the apoprotein into another species with a larger sedimentation coefficient could be demonstrated; it was actually possible to show a separation of the TPN-enzyme compound and of the free apoenzyme by ultracentrifugation (middle frames of Fig. 2). With an excess of TPN⁺, the heavier species predominated

### Table II

**Metal analysis of TPN-free glucose 6-phosphate dehydrogenase**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Analyzed sample</th>
<th>Metal detected</th>
<th>Metal</th>
<th>Metal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>µg/mg protein</td>
<td>g atom/molecule</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>&lt;0.005</td>
<td>&lt;0.013</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Ca</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>0.024</td>
<td>0.061</td>
<td>0.15</td>
</tr>
<tr>
<td>Cu</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>0.006</td>
<td>0.015</td>
<td>0.02</td>
</tr>
<tr>
<td>Fe</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>&lt;0.020</td>
<td>&lt;0.051</td>
<td>&lt;0.09</td>
</tr>
<tr>
<td>Mg</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>0.016</td>
<td>0.045</td>
<td>0.19</td>
</tr>
<tr>
<td>Mn</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>&lt;0.014</td>
<td>&lt;0.035</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>Mo</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>&lt;0.066</td>
<td>&lt;0.141</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>Co</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>&lt;0.056</td>
<td>&lt;0.141</td>
<td>&lt;0.24</td>
</tr>
<tr>
<td>Cr</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>&lt;0.056</td>
<td>&lt;0.051</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>Zn</td>
<td>Bovine serum albumin</td>
<td>&lt;0.005</td>
<td>&lt;0.010</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Bovine serum albumin</td>
<td>0.519</td>
<td>0.522 mg/ml, plus 0.498 µg Zn/ml added</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>Carboxypeptidase A</td>
<td>0.705</td>
<td>0.684</td>
<td>0.89</td>
</tr>
<tr>
<td>Cu</td>
<td>Bovine serum albumin</td>
<td>0.036</td>
<td>0.069</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* a Molecular weight of the apoprotein taken as 101,600 g (3); of bovine serum albumin 68,000; of carboxypeptidase 34,000.

* b Protein concentration of the apoprotein, 0.396 mg per ml.

* c Corrected for dialysis fluid; < implies that results were negative and values are only estimates of the sensitivity of the analysis whose signal to noise ratio varied from element to element.

* d Armour Lot 60865, 0.522 mg per ml; (0.519 - 0.005/0.498) = 103% recovery of added zinc.

* e Worthington COA DFP 6139, 0.416 mg per ml.

---

Downloaded from http://www.jbc.org by guest on November 9, 2017
Glucose 6-Phosphate Dehydrogenase. III

Vol. 244, No. 5

1.13 x 10^{-5} \text{ M TPN}^+$

6.95 x 10^{-5} \text{ M TPN}^+$

1.97 x 10^{-4} \text{ M TPN}^+$

FIG. 2. Sedimentation velocity behavior of glucose 6-phosphate dehydrogenase as a function of added TPN$. Sedimentation proceeds from right to left at 16-min intervals. Conditions: 59,780 rpm; 0.15 M KCl-0.01 M phosphate (K$^+$), pH 6.8, with varying amounts of added TPN; total protein, approximately 6 mg per ml; temperature approximately 3°. Upper: TPN$_t$ = 1.13 x 10^{-5} \text{ M}; approximately 83% light and 17% heavy component. Center: TPN$_t$ = 6.95 x 10^{-5} \text{ M}; approximately 36% light and 64% heavy component. Bottom: TPN$_t$ = 1.97 x 10^{-4} \text{ M}; approximately 10% light and 90% heavy component.

(bottom frames of Fig. 2); and finally with a huge excess (not shown here), the TPN-compound appeared to approach homogeneity. Thus, the homo- geneous TPN-free apoprotein (cf. Yue, Noltmann, and Kuby (3)) could be quantitatively converted to another species by the addition of a sufficiently high concentration of TPN$^+$. From the older data including those with excess TPN$^+$ obtained with varying ratios of light to heavy component, and consequently of varying concentrations of heavy component, values of 6.2 S and 9.5 S were estimated for $s_{20,w}$ of the light and heavy component, respectively, when extrapolated to infinite dilution (Fig. 3). The value of 6.2 S may be compared with the $s_{20,w}$ of 6.14 S for the homogeneous apoprotein (3) extrapolated from data obtained in 0.15 M NaCl-0.01 M sodium phosphate-0.01 M EDTA (or in the absence of phosphate); for comparison, these data are reproduced in the inset to Fig. 3. The satisfactory agreement for the slow component lends credence to the estimated value for $S_2$ of the fast component and to the value of $\psi$, viz. 0.657. This value for $\psi$ was assumed to apply also to the ultracentrifugal titrations (vide infra) conducted in 0.15 M NaCl, pH 6.8, where narrower concentration ranges were explored ($P_1 = 5$ to 6 mg per ml) and which, accordingly, made extrapolations more difficult for the fast component. For the older set of data, where the TPN$_t$ was relatively high compared to $P_1$ and essentially all fast component was evident, or for samples crystallized in the absence of added TPN$^+$, where essentially only slow component was present, the diffusion coefficients could be estimated from boundary spreading during centrifugation (18, 20, 29); this yielded $D_{20,w}$ values of 5.9 x 10^{-7} and 4.5 x 10^{-7} cm$^2$ per sec, respectively, for the slow and fast component. These sets of data correspond to $M_{s,p}$ values of $1.0 \times 10^4$ and $2.0 \times 10^4$ for apoprotein and TPN-compound (or polymer), respectively. Although these values were very satisfactory relative to each other, they were presented by Noltmann and Kuby (8) with the reservation that they required confirmation by a more reliable, thermodynamic technique.

The apoprotein molecular weight has since been estimated more accurately (3), first, by sedimentation velocity and translational diffusion and, second, by equilibrium sedimentation as 101,600 and 101,700, respectively, in excellent agreement with the estimate given above, which had been derived (8) from the earlier data, and which again lends support to the assigned value for $\psi$. To confirm the estimated molecular weight of the TPN-compound, low speed equilibrium sedimentation was performed at 3° (Fig. 4) in the presence of excess TPN$^+$ (approximately $1.6 \times 10^{-5} \text{ M}$) and in the absence of phosphate. This technique yielded a calculated $M_\infty$ of 2.12 x 10$^6$ (with the use of $V_\text{p} = 0.739$, measured for the apoprotein (3)) and a ratio of $M_\infty$ close to unity, attesting to the monodispersity of the heavy species. The linearity of the log $J_2$ against $X^2$ plot and the fringe deviation graph at the bottom of the figure indicate the essential absence of lighter components and point to the precision of the
determination. A slight error might have been imparted to this interferometric measurement, if a slight concentration difference existed between free TPN⁺ in the solvent and solution sectors, as a result of the binding of TPN⁺ by the protein. However, this correction cannot be significant at the total TPN⁺ concentrations used compared to the protein concentration, and the fringe deviation pattern (bottom of Fig. 4) would also imply that this error is small. The difference between this value of 2.12 × 10⁵ for Mₘ and a value of 2.06 × 10⁵ for the “dimerized” apoprotein (a molecule consisting of two apoprotein units of 101,000 and 4 moles of NaTPN⁺, vide infra) may lack significance. It is likely that the greatest uncertainty lies in the choice of V₀; the value selected for the TPN-associated species was the same as that measured for the apoprotein, viz. V₀ = 0.739. It may be calculated that, if V₀ decreased on “dimerization” by only 0.007 cm³ per g, a Mₘ of 206,000 would result.

Ultracentrifugal Titrations with TPN⁺—A number of exploratory runs were made to find suitable conditions for quantitative titrations of the apoprotein with TPN⁺, in order to approximate association constants for the dimerization reaction. The earlier studies (e.g. Fig. 2) had been conducted in the presence of dilute phosphate buffers. Some of those data are presented in Fig. 5, in terms of a plot of fraction of total protein as fast sedimenting species (i.e. Cᶠ/([Cᶠ]₀ + [Cₛ]₀)) against the mole ratio of total TPN concentration to total protein concentration expressed as apoprotein, i.e. TPN⁺/[P]₀(monomer); [P]₀(monomer) refers to concentration of total protein calculated for a molecular weight of 101,000.³ This plot permits presentation of the data from runs in which the
Fig. 6. Sedimentation velocity behavior of glucose 6-phosphate dehydrogenase as a function of added TPN+, in the absence of phosphate, at pH 6.8 (0.15 M NaCl). Temperature, 3°. Sedimentation proceeds to the right at 59,730 rpm. Photographs were taken at 10-min intervals with the schlieren diaphragm set at 75°. Concentrations of added TPN+, are given to the right of each set of frames.

Fig. 7. Ultracentrifugal titration of glucose 6-phosphate dehydrogenase with TPN+. Conditions: 3.0°; 0.15 M NaCl, pH 6.8. See text for definitions of ordinate (fraction of total protein as dimer) and abscissa (mole ratio of total TPN+ concentration to total protein concentration expressed as monomer). Other conditions were as described in the legend to Fig. 6. The solid line curve is the theoretical curve drawn for the set of equilibria postulated in the text for the TPN-induced association reactions: O——CO, titration in the absence of EDTA; □——□, titration in the presence of 0.01 M EDTA (cf. Fig. 8).

The initial total protein concentration had varied. Qualitatively, it appears that the apoprotein might be successfully titrated and that, after suitable corrections for radial dilution and for the Johnston-Ogston effect, the estimated concentrations of the two species during sedimentation—and only two species were evident—appeared to reflect the concentration distribution in solution prior to sedimentation.

Later, when liquid boundary electrophoretic studies (3) definitely indicated interactions between phosphate species and the apoprotein, and kinetic studies (4) revealed a competitive inhibition with respect to both TPN+ and glucose 6-phosphate, it was decided to explore conditions for dimer formation in the absence of phosphate or, for that matter, in the absence of added buffer species. In Fig. 6, ultracentrifugal runs are shown which were conducted at pH 6.8, 3°, in the presence of 0.15 M NaCl, at several initial concentrations of TPN+ at a fixed concentration of approximately 5.5 mg of protein per ml and in the absence of extraneous buffers. Again, an increase in initial TPN+ concentration is accompanied by a concomitant increase in the dimer concentration. The data, which had been gathered in the absence of known interacting species, are summarized graphically in Fig. 7, in the same manner as described above for Fig. 5. The data are also corrected for the Johnston-Ogston effect with the assumption that \( \psi = 0.657 \) (see "Methods") and, when compared to the data of Fig. 5, the effect of only 0.01 mM phosphate is such as apparently to increase the initial slope of the plot by a factor of about 2. Since too few data had been gathered in the presence of phosphate, below a value of \( C_{o}^{p}/[C_{o}^{p} + C_{o}^{d}] = 0.65 \), a further quantitative evaluation of this effect does not appear to be warranted.

To analyze the data of Fig. 7, the stoichiometry with respect to TPN+ of the several possible reactions must be assumed and, in addition, estimations of both bound and free TPN must be obtained from measured values for \( C_{o}^{p} \) ("dimer" or \( P_{d} \)) and \( C_{o}^{o} \) ("monomer" or \( P_{o} \)), from the known values for TPN+ and \( P_{o} \), and from an assigned value of the intrinsic association constant for the equilibrium between TPN+ and the apoenzyme.

The following are the postulated minimum number of equilibria operating in the TPN-induced "dimerization" reaction:

\[
TPN + P \xrightarrow{K_{o}} P\cdot{TPN}
\]
1.20 x 10^{-5} \text{ M TPN}^+ \\
4.74 x 10^{-5} \text{ M TPN}^+ \\
2.26 x 10^{-4} \text{ M TPN}^+

FIG. 8. Sedimentation velocity patterns of glucose 6-phosphate dehydrogenase in the presence of 0.01 M EDTA at several concentrations of added TPN+. Conditions are identical with those described for Fig. 6, except for the exact concentrations of TPN+ added; these are listed to the right of each set of frames.

The set of equilibria assumes that 1 mole of TPN+ may bind per subunit of 51,000 g, or 4 moles of TPN+ per molecule of 204,000. By assigning a $K_a$ of about $4 \times 10^4 \text{ M}^{-1}$, taken from kinetic analyses of the active "monomer," values for composite constants were approximated by curve fitting over various regions of the curve and then smoothing out over the entire curve, viz. 

$$\text{P} \cdot \text{TPN} + P \xrightleftharpoons{K_1} P_2 \text{TPN}$$

$$P_2 \text{TPN} + \text{TPN} \xrightleftharpoons{K_3} P_3 (\text{TPN})_2$$

$$P_3 (\text{TPN})_2 + \text{TPN} \xrightleftharpoons{K_4} P_4 (\text{TPN})_3$$

This set of equilibria assumes that 1 mole of TPN+ may bind per subunit of 51,000 g, or 4 moles of TPN+ per molecule of 204,000. By assigning a $K_a$ of about $4 \times 10^4 \text{ M}^{-1}$, taken from kinetic analyses of the active "monomer," values for composite constants were approximated by curve fitting over various regions of the curve and then smoothing out over the entire curve, viz. 

$$K' = K_2 K_1 = 3 \times 10^9 \text{ M}^{-2}$$

for $\text{TPN} + 2P \Rightarrow P_2 (\text{TPN})$, 

$$K' K_2 = 1.5 \times 10^{10} \text{ M}^{-2}$$

for $2 \text{TPN} + 2P \Rightarrow P_2 (\text{TPN})_2$, and 

$$K' K_2 K_3 K_4 = 5 \times 10^{14} \text{ M}^{-5}$$

for $4 \text{TPN} + 2P \Rightarrow P_4 (\text{TPN})_4$.

This analysis yielded the following estimates for the individual constants: $K_1 \approx 8 \times 10^4 \text{ M}^{-1}$, $K_2 \approx 5 \times 10^4 \text{ M}^{-1}$, and $K_3, K_4 \approx 3 \times 10^3 \text{ M}^{-3}$ (for $P_3 (\text{TPN})_2 + 2 \text{TPN} \Rightarrow P_3 (\text{TPN})_3$). $K_1$ and $K_4$ could also be approximately separated (into $3 \times 10^4$ and $1.1 \times 10^4 \text{ M}^{-1}$), but their differentiation must be considered to be within the experimental error of the measurements and the method of curve fitting.

It should be noted that the above values have been finally expressed in terms of concentration units (moles per liter) for either "monomer" or "dimer" species. The dominating terms, $K'$ and $K' K_2$, account for more than 90% of the measured curve (i.e. up to $C_\text{P}/(C_\text{P} + C_\text{TPN}) \approx 0.9$). In Fig. 7, the solid line is the theoretical curve incorporating the estimated constants; the fact that no more than 2% deviation from the experimental points is obtained over the entire plot testifies to the self-consistency of the analysis. It will be of interest to confirm the values for $n$ (maximum number of moles bound) of TPN and for $K_a$, which may be directly obtained by equilibrium-binding measurements on the "monomer," as well as the intrinsic constants for the "dimer" and to determine whether the sites for binding are equivalent.

Other mechanisms have been considered for the TPN-induced dimerization reaction, but these have been abandoned either because of lack of fit to the data or because of overcomplexity.

Preliminary measurements (J. Wu and S. A. Kuby, unpublished observations) by direct equilibrium binding with the use of Sephades G-25 (30 yl yield a value for $n$, i.e. the maximum number of moles of TPN+ bound per mole of protein, approximately equal to 2 moles of TPN+ per 101,660 g of protein, or 1 mole per subunit of 51,000 g of protein. This finding is in agreement with the conclusions drawn here.

For example, one mechanism, which could not be fitted to the data and was therefore excluded, is as follows: $P + \text{TPN} \Rightarrow \text{P} (\text{TPN})$, $2P (\text{TPN}) \Rightarrow [P_2 (\text{TPN})]$, $P_2 (\text{TPN})_2 + \text{TPN} \Rightarrow [P_3 (\text{TPN})]$, $[P_3 (\text{TPN})] + \text{TPN} \Rightarrow [P_4 (\text{TPN})]$. One mechanism, which could not be excluded, however, is the following: $P + (\text{TPN}) \Rightarrow \text{P} (\text{TPN})$, $2P (\text{TPN}) \Rightarrow [P_2 (\text{TPN})]$, $[P_2 (\text{TPN})] + \text{TPN} \Rightarrow [P_3 (\text{TPN})]$, $[P_3 (\text{TPN})] + \text{TPN} \Rightarrow [P_4 (\text{TPN})]$.
The simplest mechanism accounting for the data is the one deduced here.

Effect of EDTA on TPN-induced Association—In addition to the several buffer species which can interact with the protein (even Tris chloride to a very slight degree appears to exert some influence on "dimer" formation), EDTA at 0.01 M concentration is a rather potent inhibitor of "dimer" formation. Under conditions otherwise similar to those prevailing for the experiments shown in Fig. 6, but in the presence of a sufficiently high concentration of EDTA, separation by ultracentrifugation of the apoenzyme dimer and the TPN-enzyme is a rather potent inhibitor of "dimer" formation. Under conditions otherwise similar to those prevailing for the experiments shown in Fig. 6, but in the presence of a sufficiently high concentration of EDTA, separation by ultracentrifugation of the two species does not take place (Fig. 8), and there is only a small and perhaps insignificant effect on the calculated sedimentation coefficients. It may be inferred, therefore, that "dimerization" can be prevented by significant concentrations of EDTA.

DISCUSSION

With quantitative information on the physical parameters for the various molecular states of glucose 6-phosphate dehydrogenase now at hand, an attempt has been made to deduce a plausible model for the apoprotein and its "TPN-dimer." In Table III a summary is presented of some pertinent physical data for both species. The apoprotein of 101,600 g per mole (3) consists of two noncovalently linked subunits each of 51,000 g; thus the apoprotein is actually a dimer in itself (for definition, see Footnote 3) with a molar frictional ratio of only 1.17. In the presence of a large excess TPN, the apoenzyme apparently dimerizes again to yield a tetramer molecule with four subunits containing bound TPN, and which has an over-all molecular weight approximately twice that of the apoprotein. It is notable (cf. Table III) that the frictional ratios reveal only very slight differences between the apoenzyme dimer and the TPN-enzyme tetramer, viz. 1.17 and 1.19, respectively. These values would correspond to axial ratios of 3.6 and 4.1 for the apoprotein and the TPN species, respectively, for an assumed anhydrous prolate ellipsoid of revolution. These data would seem to preclude that a "head to tail" combination could occur in the association process induced by TPN+. This assumption, of course, does not consider the possibility that the hydrodynamic changes caused by TPN may be accompanied by additional conformational changes not revealed in the gross molecular changes.

Since the $f/b$ values remain essentially constant in the association process, a plausible prolate ellipsoid model to allow for only this fact would provide an axial ratio of approximately 1.5 (or multiples thereof) for both the hydrated species of the apoprotein and its associated TPN-product. Thus, for the tetrameric TPN-compound, this allows calculation from Perrin's equation (31) of $(f/b)_{\text{apoenzyme}} \approx 1.03$, and therefore

$$
(f/b)_{\text{apoenzyme}} = \frac{(f/b)_{\text{eqv}}}{(f/b)_{\text{hydration}}} = \frac{1.19}{1.152} \approx 1.03
$$

and $W \approx 0.398$ g H$_2$O per g of protein as the water of hydration.

The molar volume of the hydrated TPN-species may be computed next as $V_{\text{m}}} {\text{hydrated}} = M \bar{V} [1 + (W/M_\text{pH})] \approx 235$ liters per mole (for $M = 2.06 \times 10^5$, see above). For a prolate spheroid, $V = 4/3 \pi a b^2$; setting $a/b$ (for major to minor axis) $\approx 1.5$, $V = 2 \pi b^2$; finally substituting the volume per molecule (39.1 $\times 10^{-26}$ cm$^3$ per molecule) yields a value of $b \approx 39.8$ Å, and consequently $a = 59.5$ Å.

Such a prolate spheroid model for the four-subunit TPN-enzyme compound may be visualized as drawn in Fig. 9 in a two dimensional plane. As discussed above, a head to tail association between half-molecules is not invoked. Rather, a half-molecule contains two subunits, each aligned such that they lie parallel to their individual major axes and in contact; the major axis of the individual subunit lies perpendicular to the major axis of the prolate ellipsoid of the apoenzyme or half-molecule. The combination of two such half-molecules proceeds in the presence of TPN+ such that their contact surfaces lie along the minor axis of the final tetrameric model. The major axes of the apoenzyme prolate ellipsoids, in passing from the dimeric to tetrameric species, are oriented parallel to the minor axis of the

![Figure 9](http://www.jbc.org/)

**Fig. 9. Hypothetical model for the TPN-induced, solvated structure of glucose 6-phosphate dehydrogenase in the tetra-chain form.** The model is not drawn to scale, but has been exaggerated in its dimensions and relative proportions in order to emphasize the contribution of the water of hydration to the structure and to show the change in orientation axes on transformation of the two-chain apoprotein species to the tetra-chain TPN-species. The anhydrous molar volume of the tetrameric TPN-species ($M_\text{V} \approx 2.06 \times 10^5 \times 0.744 - 153$ liters per mole) may actually be calculated to be 65.2% by volume of the final model containing 39.8% water by weight (i.e. $V_{\text{m} \text{h}} \text{hydrated} \approx M_\text{V} [1 + (W/M_\text{pH})] = 2.06 \times 10^5 (0.744 + 0.398) - 235$ liters per mole, see text).
that the interaction of EDTA with the enzyme is unrelated to the chelation properties of EDTA, since it has been shown that the protein itself does not contain significant concentrations of zinc, or of other divalent cations likely to be present. Thus, the explanation for the ability of significant concentrations of EDTA to inhibit the TPN-induced association process must be sought elsewhere. Moreover, wherein lies the ability of EDTA to induce the dimerization reaction? Undoubtedly, specific interactions between TPN and sites on the enzyme molecule occur, thus shifting the "monomer"-"dimer" equilibrium. Thermodynamically, the protein association reactions can be expressed in terms of a set of equilibria involving the successive binding of a total of 4 moles of TPN to the four-subunit model. However, it is difficult to invoke a competition for specific "sites," including catalytic sites, between TPN and EDTA, since the latter bears no chemical or structural similarity to TPN. The binding of TPN per se may also modify solvent-macromolecule interactions and it is of course likely that the preferentially bound solvent itself is intimately involved in the final formation of the associated structure. The close domain solvent structure of the "monomer" hydrated water ("side supra") would thus be altered or perturbed by the binding of TPN to specific amino acid residues on the protein molecule, so as to permit a new orientation in the structure of the bound water in the final TPN-induced "dimer" hydrated molecule (i.e. the hydrated tetra-subunit model). Thus, TPN itself on binding could release electrons to water at an ionic binding site to the extent of possibly about 20 ml per mole of dipole (see Cohn and Edsall (32)). If 4 moles of TPN were bound in the final association process, as much as 80 ml per 2 $\times$ 10$^{-9}$ g of protein could be released. Admittedly, this would make an insignificant total change in, for example, an estimated molar volume of 253 liters ("side supra"); however, the release of such water from each subunit and, in turn, the binding of TPN with the masking of an ionic group would then require a restabilization of the surrounding solvent layer so as to stabilize whatever linkages or forces are involved at the contact surfaces between the half-molecules to yield the tetra-subunit model. Thus, EDTA could prevent "dimerization" by affecting or further stabilizing the structures of the surrounding bulk solvent via producing a new "structure-making effect" in the solvent which in turn would modify the solvent macromolecular interaction involved in the formation of the associated structure.

Shalaby and Lauffer (10) have also found that added solutes of varying chemical nature affected the polymerization of tobacco mosaic virus protein. Interestingly, EDTA at significant concentrations also retarded the polymerization process. They rejected the chelating action of EDTA as the responsible factor and brought forward a plausible hypothesis involving an effect resulting from its action on the solvent. Frank (33) has summarized and reviewed the current ideas (e.g. References 34 to 38) about the possible structures of water and aqueous solutions and the possible alternatives according to which solutes of varying chemical nature might influence the solvent structure. Since multivalent ions have been found to increase the viscosity of water, they have been considered to have a net structure-making effect. On this basis, a competition between two types of processes may be proposed: the structure-making effect of the surrounding solvent by EDTA$^{2-}$ and the solvent-protein interactions. Thus, EDTA$^{2-}$ could prevent the TPN-induced association effect by modifying the structure of the solvent; such a modification could in turn perturb interactions between the solvent and protein macromolecule so as to destabilize the "dimer." Thus, EDTA would exert its effect indirectly on the TPN-induced macromolecular association behavior. Such an interpretation is also in accord with the reasoning of Timasheff and Issue (39) who discussed preferential binding of solute components to lysozyme, albumin, and insulin in terms of the affinities of different amino acid residues for various types of media as the protein conformation is altered by the change in the medium.

The sum total of all linkages or forces involved in stabilizing the tetra subunit model containing TPN can only be guessed at. One clue, of course, is the fact that the TPN-compound may be induced to crystallize at very high ionic strengths by the addition of (NH$_4$)$_2$SO$_4$ to a 2.5% protein solution in 0.01 M TPN, to yield a final concentration of (NH$_4$)$_2$SO$_4$ close to 2.3 M (1). Moreover, the solubility of the crystalline apoprotein (or half-molecule) is greater than the crystalline TPN-compound in (NH$_4$)$_2$SO$_4$ solutions. Ionic forces at the contact surfaces between associated half-molecules may be de-emphasized in the final TPN-compound, but both hydrophobic and hydrogen bonding must play a significant role in order to permit a stable and ordered macromolecular structure for the TPN species to exist at these high ionic strengths.

With these comments, it may be predicted that diverse chemical compounds can exert varied influences on the association-dissociation equilibria of this enzyme, and that the participation of TPN is neither unique, nor that the destabilizing effect of EDTA is unusual.

Acknowledgments—We are indebted to Dr. Norman Weissman of the University of Utah, Departments of Pathology and Biochemistry, for permission to use his atomic absorption equipment and for his advice and his assistance with the metal analyses. We also wish to acknowledge the excellent technical assistance of C. M. Richardson and L. J. Maland. Finally, the authors wish to thank Anheuser-Busch, Inc., and particularly Dr. R. Seeley and Mr. M. L. Dackels, for generous quantities for their dried brewers' yeast.

REFERENCES

Glucose 6-Phosphate Dehydrogenase. III

Glucose 6-Phosphate Dehydrogenase from Brewers' Yeast (Zwischenferment) : III. STUDIES ON THE SUBUNIT STRUCTURE AND ON THE MOLECULAR ASSOCIATION PHENOMENON INDUCED BY TRIPHOSPHOPYRIDINE NUCLEOTIDE

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

J. Biol. Chem. 1969, 244:1353-1364.

Access the most updated version of this article at http://www.jbc.org/content/244/5/1353

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/244/5/1353.full.html#ref-list-1