A STUDY OF THE MECHANISM BY WHICH TRIPHOSPHOPYRIDINE NUCLEOTIDE AFFECTS HUMAN ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE[1]

Ethel A. Tsutsui and Paul A. Marks

Department of Medicine, Columbia University
College of Physicians and Surgeons, New York City

Received June 20, 1962

Triphosphopyridine nucleotide (TPN) or TPNH has been found to protect glucose-6-phosphate (G6P) dehydrogenase against inactivation (Kirkman, 1959; Marks et al., 1959). Recently it was shown that purified preparations of G6P dehydrogenase can be activated by TPN or TPNH with an accompanying alteration in the electrophoretic mobility of the enzyme (Marks, 1961). These findings suggested that the enzyme may exist in different molecular forms. Evidence has now been obtained to indicate that the stabilization and activation of G6P dehydrogenase in the presence of TPN is associated with a molecular alteration in the protein characterized by an increase in its sedimentation velocity and in its catalytic activity relative to its antigenic activity.

METHODS: G6P dehydrogenase was initially purified from erythrocytes of human subjects by a method described elsewhere (Marks et al., 1961) which yields an ammonium sulfate fraction which is approximately 500-fold purified. The enzyme has now been purified further by treating this ammonium sulfate fraction with Sephadex, diethylaminoethyl cellulose column chromatography and ammonium sulfate fractionation yielding a preparation about 5000-fold purified with a recovery of 15 to 20 per cent. The best specific activity obtained was 20 units/mg protein (a unit of enzyme activity is defined as a change of 1.0 optical density unit/mg protein under the assay conditions described by Marks et al., 1961). All the present studies were performed with this ammonium sulfate fraction.

The technique of Martin and Ames (1961) was employed to study the sedimentation behavior of the enzyme in the presence and absence of TPN. For these studies identica

1. This research was supported in part by Grants RG 7368 and CY 2332 of the U.S. Public Health Service.

aliquots of the purified enzyme preparation were placed in the centrifuge tube on top of a sucrose gradient without TPN or containing TPN, 4 x 10^{-4}M. Purified hemoglobin and/or crystalline alcohol dehydrogenase were added as markers and centrifuged as indicated in Figure 1. The two marker proteins centrifuged to the same relative position in the presence and absence of TPN. Following centrifugation, fractions of the gradient were assayed for (1) G6P dehydrogenase activity; (2) total protein cross-reacting with antibody to G6P dehydrogenase and (3) hemoglobin and/or alcohol dehydrogenase activity. The cross-reacting protein was determined by a method similar to that of Perrin et al., (1959). For this purpose, antisera inhibitory to G6P dehydrogenase were produced in rabbits by injection of a Freund adjuvant preparation containing the enzyme and TPN, 4 x 10^{-4}M (Tsutsui and Marks, 1962). The assay system

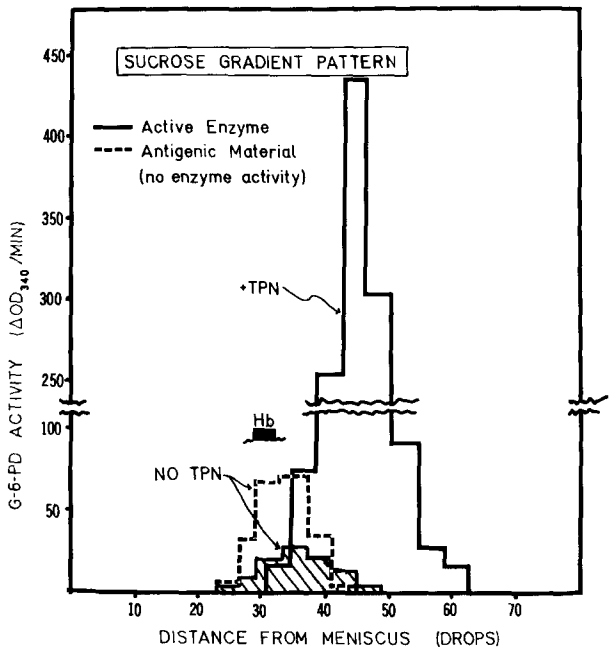

Figure 1

Recovery of catalytically active G6P dehydrogenase (active enzyme) and protein cross-reacting with antibody to G6P dehydrogenase but without catalytic activity (antigenic material) after centrifugation in sucrose gradients in the presence of TPN, 4 x 10^{-4}M (+TPN) and in the absence of TPN (no TPN). 0.1 ml. of Tris(hydroxymethyl)aminomethane buffer, 0.05 M, pH 7.6, containing 2870 units of G6P dehydrogenase, and 12 mg of hemoglobin with or without TPN was layered on top of sucrose gradients. Centrifugation was for 17 hours at 35,500 r.p.m. in an SW-39 rotor in a Spinco model L centrifuge. After centrifugation, fractions were collected as described by Martin and Ames (1961). The cross-reacting protein is expressed in terms of units of G6P dehydrogenase as defined in the text.

for the cross-reacting protein was as follows: an aliquot of each fraction from the gradient was added to each of two tubes, one containing 0.02 ml control serum (serum from rabbit obtained prior to immunization), 200 units of G6P dehydrogenase, 4 x 10^{-4}M TPN, and 0.05 M Tris buffer, pH 7.4 in a final volume of 0.5 ml., the other containing the same mixture but with 0.02 ml. antiserum rather than control serum. The amount of G6P dehydrogenase (200 units) represents a slight excess of antigen for the amount of antiserum employed. After an incubation for 18 hours at 4°C, the tubes were centrifuged and the G6P dehydrogenase activity in the supernatant fluid determined. The amount of cross-reacting protein was expressed in terms of units of G6P dehydrogenase activity recovered in the antiserum tube in excess of that expected on the basis of 1) the amount of active enzyme in the fraction of the gradient plus 2) the enzyme activity remaining uninhibited in an antiserum tube with no added fraction from the gradient.

RESULTS AND DISCUSSION: The sedimentation velocity of catalytically active G6P dehydrogenase was greater in the presence than in the absence of added TPN (Figure 1). In the absence of TPN, only 5% of the catalytically active enzyme was recovered following centrifugation (Table 1). However, under conditions of no added TPN, immunochemically cross-reacting material without catalytic activity was present and was found to coincide in its sedimentation velocity with that of the catalytically active

Table 1. Effect of TPN on sedimentation behavior of G6P dehydrogenase

Addition	Units of enzyme activity[1] Applied	Recovered	R_{Hb}[2]	$R_{A.D.}$
None	3760	188	1.13	0.69
TPN 4 x 10^{-4}M	3810	3240	1.35	0.85

1. These figures represent units of enzyme activity applied to the sucrose gradient and the units of enzyme activity recovered in all fractions of the gradient following centrifugation.

2. $R = \frac{\text{Distance travelled from meniscus by G6P dehydrogenase}}{\text{Distance travelled from meniscus by marker}}$

Abbreviations: Hb, hemoglobin; A.D., alcohol dehydrogenase.

enzyme (Figure 1). In the gradient run in the presence of TPN, all the antigenically active material was catalytically active, i.e., no cross-reacting protein without catalytic activity was detectable (Figure 1). In all experiments, single peaks for the active enzyme and for the cross-reacting material were obtained.

The loss in enzyme activity which occurs in the absence of TPN appears to be associated with the conversion of G6P dehydrogenase, in part, to a form which has a lower catalytic activity per unit of antigenic activity. Since the enzyme preparation employed is not pure, it is not possible to determine if TPN alters the antibody combining capacity per mg of enzyme protein. In the absence of TPN, cross-reacting protein plus catalytically active G6P dehydrogenase recovered accounted for only 13% of the protein applied to the gradient. This apparent poor recovery of enzyme protein in the absence of TPN could reflect either an alteration in the antibody combining capacity of the protein or a denaturation of a major portion of the enzyme associated with a loss in both antigenic and catalytic activity. It has previously been demonstrated (Marks *et al.*, 1961) that treating G6P dehydrogenase with mild heat or dilution in the absence of TPN causes an inactivation of the enzyme which is not reversed by addition of high concentrations of TPN, suggesting that this loss in activity involves an irreversible denaturation of the protein.

The present data indicate that removal of TPN alters the molecular structure of G6P dehydrogenase and that the presence of TPN is important for the preservation of the native configuration of this protein. Nirenberg and Jakoby (1960) have previously provided evidence that the combination of pyridine nucleotide with the enzyme succinic semialdehyde dehydrogenase is associated with an altered configuration of the enzyme. The present data do not permit conclusions as to the nature of the molecular alteration in G6P dehydrogenase induced by TPN. This must await investigation with a more highly purified preparation of the enzyme.

REFERENCES

Kirkman, H.M., Nature, 184, 1291 (1959)
Marks, P.A., in Cold Spring Harbor Symposia on Quantitative Biology, 26, 343 (1961)
Marks, P.A., Banks, J. and Gross, R.T., Biochem. Biophys. Res. Comm. 1, 199 (1959)
Marks, P.A., Szeinberg, A. and Banks, J., J. Biol. Chem. 236, 10 (1961)
Martin, R.G. and Ames, B.N., J. Biol. Chem. 236, 1372 (1961)
Nirenberg, M.W. and Jakoby, W.B., Proc. Natl. Acad. Sci., 46, 206 (1960)
Perrin, D., Bussard, A. and Monod, J., Comptes Rendus, 249, 778 (1959)
Tsutsui, E.A. and Marks, P.A., Fed. Proc. 21, 253 (1962)