Purification and Properties of Chicken Liver D-3-Phosphoglycerate Dehydrogenase*

D. A. Walsh and H. J. Sallach

ABSTRACT: D-3-Phosphoglycerate dehydrogenase from chicken liver has been purified extensively by fractionation with ammonium sulfate and by chromatography on diethylaminoethyl-, carboxymethyl-, and phosphocelluloses. The initial reaction velocities have been determined spectrophotometrically at *p*H 8.0. From the double-reciprocal plots, the Michaelis constants for all four substrates and the dissociation constants for oxidized and reduced nicotinamide–adenine dinucleotide (NAD^+ and NADH) have been determined. The reaction of the enzyme with analogs of NAD^+ and with reduced nicotinamide–adenine dinucleotide phosphate (NADPH) has been investigated. The enzyme is inhibited by *N*-ethylmaleimide, 3-bromopyruvate, and organic mercurials. Preincubation of the enzyme with NADH or NAD^+, but not with phosphoglycerate, partially protects the enzyme against inhibition by the first two reagents.

D-3-Phosphoglycerate dehydrogenase catalyzes the formation of phosphohydroxypyruvate from D-3-phosphoglycerate. The occurrence of this enzyme in mammalian systems was first reported by Ichihara and Greenberg (1957), who demonstrated the formation of phosphoserine and serine from phosphoglycerate by an enzyme preparation from rat liver. A similar conversion has been shown in an enzyme system from pea epicotyls (Hanford and Davies, 1958). Phosphoglycerate dehydrogenase has been demonstrated in extracts of *Salmonella typhimurium* (Umbarger and Umbarger, 1962) and *Escherichia coli* (Pizer, 1963), and has been shown to be sensitive to inhibition by L-serine. The widespread occurrence of phosphoglycerate dehydrogenase, as well as D-glycerate dehydrogenase, in vertebrate tissues was reported in an earlier communication from this laboratory (Willis and Sallach, 1964). The former enzyme was purified 5-fold, and the reaction products of the oxidation of phosphoglycerate and the reduction of phosphohydroxypyruvate by diphosphopyridine nucleotides were characterized.

The purpose of this paper is to present the procedure for the first extensive purification of phosphoglycerate dehydrogenase and the determination of the kinetic constants of the reaction. The nucleotide specificity, inhibition studies, and the effects of other metabolites on the activity of the enzyme are also reported.

Materials and Methods

Substrates. The potassium salt of D-3-phosphoglycerate was prepared from the barium salt (Sigma Chemical Co.) as described previously (Willis and Sallach, 1962). The cyclohexylammonium salt of the dimethyl ketal of phosphohydroxypyruvate (California Corp. for Biochemical Research) was converted to the free acid as outlined by Ballou (1960). All nucleotides, including the analogs of NAD^+,[1] were purchased from Pabst Laboratories. The concentrations of the nucleotide solutions used were determined from their respective molar extinction coefficients (Horecker and Kornberg, 1948; Siegel *et al.*, 1959).

Dalziel (1963) has shown that some commercial preparations of NAD^+ contain small amounts of impurities which, at lower *p*H values, markedly affect the initial reaction rates of certain dehydrogenases and hence the kinetic constants. Therefore, for control experiments NAD^+ was purified by chromatography on DEAE-cellulose as described by Dalziel (1963). Essentially the same procedure was used for the purification of $NADP^+$ with the following modification. The mixing vessel for the gradient elution contained 500 ml of 0.045 M potassium phosphate buffer, *p*H 6.0, and the buffer in the reservoir used for the concentration gradient was 0.3 M potassium phosphate, *p*H 6.0; other conditions were identical. $NADP^+$ was eluted at a concentration of 0.23 M phosphate by this procedure. The initial reaction rates and the Michaelis constants obtained with the purified NAD^+ at *p*H 8.0 did not vary significantly from the values determined with the original sample. Similarily, no difference in reaction rates was observed with purified and nonpurified $NADP^+$. In view of these

* From the Department of Physiological Chemistry, University of Wisconsin Medical School, Madison. *Received January 25, 1965.* This investigation was supported in part by a research grant (AM 00922-10) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service, and in part by the Wisconsin Alumni Research Foundation.

[1] Abbreviations used in this work: NAD^+, NADH, $NADP^+$, NADPH, the oxidized and reduced forms of nicotinamide–adenine dinucleotide and nicotinamide–adenine dinucleotide phosphate, respectively; NEM, *N*-ethylmaleimide; GSH, glutathione; AMP, ADP, and ATP, adenosine 5′-mono-, di-, and triphosphates, respectively.

results, the commercial preparations of the nucleotides were used routinely.

Other Materials. 3-Bromopyruvate was prepared from pyruvate by the method of Sprinson and Chargaff (1946). The organic mercurials, iodoacetate (recrystallized from 95% ethanol), and NEM were commercial preparations (Sigma Chemical Co.). Dithiothreitol was prepared by Dr. Collin Schroeder, Wisconsin Alumni Research Foundation, by the procedure of Evans *et al.* (1949). D-Glycerate and hydroxypyruvate were obtained as outlined elsewhere (Willis and Sallach, 1962). *O*-Phospho-L-serine was synthesized according to the method of Neuhaus and Korkes (1958). The ammonium salt of carbamylphosphate was prepared as described by Metzenberg *et al.* (1960). Other compounds used in these studies were commercial preparations.

The method of Cori *et al.* (1948) was used to prepare crystalline rabbit muscle triosephosphate dehydrogenase. Yeast 3-phosphoglycerate kinase and glucose-6-phosphate dehydrogenase were purchased from C. F. Boehringer und Soehne GmbH, Mannheim. Highly purified carbamylphosphokinase from *Streptococcus faecalis* was a gift of Drs. M. Marshall and P. P. Cohen of this department. DEAE-cellulose, CM-cellulose, and phosphocellulose were purchased from Eastman Organic Chemicals, Sigma Chemical Co., and Bio-Rad Laboratories, respectively.

Standard Assay for Phosphoglycerate Dehydrogenase. One unit of activity was defined as that amount of enzyme that produced an increase in absorbance of 0.001/min at 340 mμ under the conditions described. (This unit is equivalent to the formation of 3.61×10^{-4} μmole of NADH per minute.) The specific activity equals the number of units per mg of protein. The 2.25-ml incubation mixture contained: Tris-chloride buffer, *p*H 9.0, 500 μmoles; hydrazine acetate, *p*H 9.0, 400 μmoles; NAD^+, 1.5 μmoles; GSH, 5 μmoles; EDTA, 25 μmoles; D-3-phosphoglycerate, 25 μmoles (omitted from the control); and the enzyme preparation to be tested. The reaction was started at 25° by the addition of NAD^+ and followed by measurement of the absorbance at 340 mμ over a 5-minute period with a Beckman DU spectrophotometer.

Method for the Identification of Phosphoglycerate Produced by the Enzymatic Reduction of Phosphohydroxypyruvate with NADPH. The oxidation of NADPH was measured spectrophotometrically in a system containing: sodium bicarbonate, *p*H 6.9, 80 μmoles; glycine, *p*H 6.9, 107 μmoles; phosphohydroxypyruvate, 0.387 μmole; NADPH, 0.166 μmole; and 0.075 mg of phosphoglycerate dehydrogenase in a total volume of 2.3 ml. NADPH was omitted from the complete system (NADPH blank) and was used as a control in the assay described. After the total oxidation of NADPH in the complete system, the enzyme was destroyed by heating the solutions at 100° for 3 minutes.

The above-mentioned solutions were assayed by an adaptation of the procedure described by Bucher (1955) for phosphoglycerate kinase, which employs triosephosphate dehydrogenase and the spectrophotometric determination of NADH disappearance. Phosphoglycerate assays were carried out on 1-ml aliquots of the complete system and of the NADPH blank. The latter was used as a control for the nonspecific removal or breakdown of NADH. To each of the 1-ml aliquots was added: ATP, 0.1 μmole; magnesium sulfate, 10 μmoles; cysteine (freshly prepared), *p*H 6.9, 20 μmoles; carbamylphosphate, 10 μmoles; NADH, 0.195 μmole; in a total volume of 2.25 ml. The initial optical density was recorded and the reaction was started by the addition of 0.025 mg of phosphoglycerate kinase, 0.24 mg of triosephosphate dehydrogenase, and 0.065 mg of carbamylphosphokinase to give a final volume of 2.35 ml. The amount of NADH oxidized gave a measure of phosphoglycerate in the aliquot. The equilibrium of the phosphoglycerate kinase reaction lies in the direction of D-3-phosphoglycerate formation. The addition of carbamylphosphate and carbamylphosphokinase results in the regeneration of ATP with the removal of ADP; this promotes the formation of 1,3-diphosphoglycerate and the complete conversion of D-3-phosphoglycerate. A control system, in which phosphohydroxypyruvate was omitted in the original incubation and subsequently analyzed for phosphoglycerate, gave results equivalent to the NADPH blank.

Other Analytical Methods. For the enzyme purification, protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. All other protein determinations were calculated from the absorbance at 280 mμ, assuming $E^{1\%}_{1\,cm} = 9.0$ (Warburg and Christian, 1942). A Radiometer No. 4 *p*H meter was used for *p*H measurements.

Kinetic Measurements. All kinetic experiments reported here were performed at 25° in a cuvet with a light path of 1 cm. The initial linear reaction velocities (at least 60 seconds, depending on conditions) were determined from the absorbance changes at 340 mμ with the 80–100% expanded scale of a Honeywell recorder used in conjunction with a Beckman DU spectrophotometer. The slit width was held at less than 0.3 mm.

The stock enzyme solution was diluted from a saturated ammonium sulfate suspension for each series of experiments (3–4 hours) and was stored at 0° in 0.05 M potassium phosphate buffer, *p*H 6.2, containing 0.05 M β-mercaptoethanol and 1.0 M sodium chloride, over the duration of each experiment. Assays were carried out at *p*H 8.0 in 0.25 M Tris-chloride buffer containing 2.5×10^{-3} M GSH and 1.25×10^{-2} M EDTA. The addition of the enzyme to the cuvet initiated the reaction and resulted in a final concentration of 0.1 M sodium chloride and 5×10^{-3} M β-mercaptoethanol in the reaction mixture. Because of the unfavorable equilibrium constant, the reactions in the direction of phosphohydroxypyruvate formation were performed in the presence of 1.0×10^{-2} M semicarbazide analogous to the method used by Winer and Schwert (1958) with lactate dehydrogenase. Control experiments showed that 0.01 M semicarbazide had no significant effect on the initial reaction velocity.

The susceptibility of the enzyme to inactivation was monitored by the repeated use of standard assays over

the period of each experiment. In the direction of pyridine nucleotide oxidation, the standard incubation system contained 12.5×10^{-5} M phosphohydroxypyruvate and 1.6×10^{-5} M NADH. In the reverse direction, the standard contained 8×10^{-4} M NAD^+ and 8×10^{-3} M phosphoglycerate. The enzymatic activity was corrected back to zero-time activity and only those data from experiments in which the total denaturation over the complete experimental period (3–4 hours) was less than 15% of the maximum activity were used. The kinetic data in Figures 3–6 have all been corrected to approximately the same enzyme concentration and represent the results of several determinations.

Enzyme Stabilization. During the course of this investigation, a new protective reagent for thiol groups, dithiothreitol, was introduced by Cleland (1964). This substance was found to be extremely useful in counteracting the instability of phosphoglycerate dehydrogenase. Preincubation of the enzyme with dithiothreitol followed by the removal of this compound, as outlined later, resulted in an enzyme that was relatively stable in the absence of any protective reagents for thiol groups. The enzyme used for the inhibition studies with NEM, 3-bromopyruvate, and organic mercurials was treated in the following manner: Protein (2.5–5.0 mg) was incubated at 25° for 30 minutes with 10 μmoles of dithiothreitol, 160 μmoles of potassium phosphate, *p*H 7.4, 160 μmoles of sodium chloride, and 2.4 μmoles of EDTA in a final volume of 1 ml. The solution was then applied to a Sephadex G-25 column (27 × 1.0 cm) equilibrated with 0.2 M potassium phosphate buffer, *p*H 6.0, containing 0.2 M sodium chloride and 3×10^{-3} M EDTA. The column was eluted with the same buffer. The eluted protein was diluted in the same buffer to a concentration of not less than 0.3 mg/ml. Enzyme solutions prepared in this manner may be stored at 0° for at least 5 hours without significant loss of activity. More concentrated solutions of the enzyme were found to be stable for much longer periods of time.

Results

Purification of Phosphoglycerate Dehydrogenase from Chicken Liver. The following general conditions were used in the purification of the enzyme unless otherwise specifically indicated. All operations were conducted at 4°. Fractionations with ammonium sulfate were made by the slow addition, with stirring, of the calculated amount of solid ammonium sulfate. The resulting suspensions were equilibrated for 30 minutes before centrifugation. Centrifugations were carried out in a Servall RC-2 centrifuge at 14,000 × *g* for 25 minutes. In the chromatographic procedures employing cellulose derivatives, protein in the eluates from the columns was detected by the absorbance at 260 mμ.

A typical enzyme preparation was made according to the following procedure: Chicken liver (2.13 kg) was homogenized in a Waring Blendor for 1 minute with 4.26 liters of isotonic KCl (*p*H 7.4). The supernatant solution (fraction A) obtained on centrifugation was adjusted to *p*H 4.6 by the slow addition of 1 N acetic acid. The suspension was equilibrated with stirring for 15 minutes. The precipitate was removed by centrifugation and discarded. The following alternative procedure for these two steps was established and used in later studies. The liver was homogenized, as before, in two volumes of 0.15 M potassium acetate buffer, *p*H 4.6, containing 3×10^{-3} M EDTA and 1×10^{-3} M β-mercaptoethanol, and the homogenate was centrifuged. To the supernatant solution (fraction B) at *p*H 4.6 were added 24 mmoles of EDTA, *p*H 6.0, and 3.5 mmoles of β-mercaptoethanol to give a final volume of 3.53 liters. The solution was brought to 43% saturation with 954 g of ammonium sulfate. The precipitate obtained on centrifugation was resuspended in 353 ml of 5×10^{-3} M EDTA containing 2.0×10^{-3} M β-mercaptoethanol (0.1 of the original volume). The residue obtained on centrifugation was discarded and the supernatant fluid (fraction C) was adjusted to *p*H 7.2 by the addition of 465 ml of 0.5 M potassium phosphate buffer, *p*H 7.8 (0.5×10^{-3} M β-mercaptoethanol and 3×10^{-3} M EDTA), and was brought to 21% saturation with 111.6 g of ammonium sulfate. The ammonium sulfate precipitate was removed by centrifugation and the supernatant solution was brought to 41% saturation with 111.6 g of ammonium sulfate. The precipitate was recovered by centrifugation and could be stored at −15° for several days.

All buffers used in the remaining steps of the purification contained 0.5×10^{-3} M β-mercaptoethanol and 3×10^{-3} M EDTA except where otherwise stated. The 21–41% ammonium sulfate precipitate was dissolved in 200 ml of 0.01 M potassium phosphate buffer, *p*H 6.0, and the resulting solution was dialyzed against the same buffer with regular changes until the dialysate gave a negative reaction to Nessler's reagent (approximately 6 hours). The enzyme solution (fraction D) was adjusted to *p*H 7.6 with 0.3 M ammonium hydroxide and applied to a DEAE-cellulose column (14 × 6 cm) equilibrated with 0.01 M potassium phosphate buffer, *p*H 7.6. The DEAE-cellulose column was washed with the same buffer (flow rate, 440 ml/hr). The first 680 ml of solution, after the hold-up volume, was collected (fraction E) and adjusted to *p*H 5.6 with 1 N acetic acid. The enzyme solution was applied to a CM-cellulose column (40 × 6 cm) equilibrated with 0.01 M potassium phosphate buffer, *p*H 6.0. The column was washed with the same buffer (flow rate, 900 ml/hr) until the eluate was free of protein. The column was eluted with 0.01 M potassium phosphate buffer, *p*H 6.9, and the protein in this fraction was discarded. The enzyme was eluted from the column with 0.025 M potassium phosphate buffer, *p*H 7.4 (fraction F). To the 500 ml of enzyme solution was added 250 ml of 0.1 M potassium phosphate buffer, *p*H 6.4. The solution was adjusted to *p*H 6.0 with 1 N acetic acid and applied to a CM-cellulose column (14 × 4.5 cm) equilibrated with 0.01 M potassium phosphate buffer, *p*H 6.0. The column was washed with 0.05 M potassium phosphate buffer, *p*H 6.0; 800 ml of enzyme solution was collected (fraction G) and was brought to 62% saturation with 320 g of ammonium sulfate. The precipitated protein was recovered by cen-

trifugation and could be stored at −15° for several days.

The ammonium sulfate residue was dissolved in 0.01 M potassium phosphate buffer, *p*H 6.0, to give a final volume of 35 ml, and the resulting solution was applied to a Sephadex G-25 column (54 × 4.6 cm) equilibrated with 0.01 M potassium phosphate buffer, *p*H 6.0. The column was washed with the same buffer; the protein solution, collected in a volume of 110 ml, gave a negative reaction to Nessler's reagent. The protein solution was applied to a phosphocellulose column (15.5 × 4.7 cm) equilibrated with 0.01 M potassium phosphate buffer, *p*H 6.0. The column was washed with an additional 50 ml of the same buffer and eluted with a gradient of phosphate concentration. The constant-volume mixing vessel contained 350 ml of 0.01 M potassium phosphate buffer, *p*H 6.0, and the reservoir contained 0.3 M potassium phosphate buffer, *p*H 6.0. Phosphoglycerate dehydrogenase was eluted at a molarity of 0.12 M potassium phosphate in the fractions of the first protein peak (fraction H). The maximum specific activity of an individual fraction was 20,000 and the average specific activity of the pooled fractions, containing 79% of the enzyme applied to the column, was 15,600. (It was established later that the direct elution of the phosphocellulose column with 0.13 M potassium phosphate buffer, *p*H 6.0, results in an enzyme preparation with a specific activity of 14,000 and a yield of 79% of the enzyme applied.) The eluate containing phosphoglycerate dehydrogenase (88 ml) was brought to 62% saturation by the addition of 35.2 g of ammonium sulfate. The precipitate was recovered by centrifugation and could be stored at −15° for several weeks with little loss in activity. For shorter time periods, the enzyme may be stored as a suspension in saturated ammonium sulfate at 4°. This enzyme preparation was used in the studies reported here. A summary of the purification data is presented in Table I.

TABLE I: Summary of Enzyme Purification Data.

Enzyme Fraction	Specific Activity	Per Cent Recovery
A	22	
B	88	100
C	302	100
D	480	92
E	671	88
F	4,360	71
G	5,595	51
H	15,600	40

Specificity of the Enzyme. Phosphoglycerate dehydrogenase, as originally described (Willis and Sallach, 1964), was shown to react with nicotinamide–adenine dinucleotides. This specificity has been found not be to absolute. A lesser reactivity with nicotinamide–adenine dinucleotide phosphates, as described later, has been demonstrated. In the standard assay system, purified phosphoglycerate dehydrogenase had an initial rate of production of NADH of 2 μmoles/min per mg of protein. Using identical concentrations of phosphoglycerate, coenzyme, and enzyme, no reaction was observed with $NADP^+$. If the phosphoglycerate concentration was increased 8-fold (final concentration, 0.089 M) and the coenzyme concentration was increased 3-fold (final concentration, 2×10^{-3} M), the rate of production of NADPH was 1.5×10^{-2} μmole/min per mg of protein. The reverse reaction was assayed by the disappearance of reduced pyridine nucleotide as determined spectrophotometrically. The assay system contained Tris-chloride buffer, *p*H 8.0, 500 μmoles; EDTA, 25

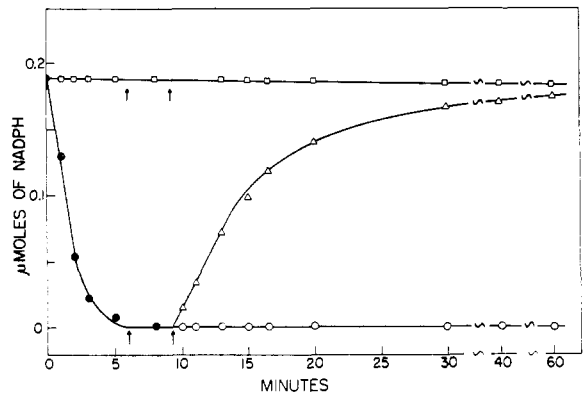

FIGURE 1: The identification of $NADP^+$ as the product of phosphohydroxypyruvate reduction with NADPH and phosphoglycerate dehydrogenase by assay with yeast glucose-6-phosphate dehydrogenase. The initial reaction mixture in each of three cuvets contained glycylglycine buffer, *p*H 7.4, 250 μmoles; EDTA, 25 μmoles; and NADPH, 0.188 μmole. Phosphohydroxypyruvate, 0.28 μmole, was added to two cuvets (complete system, ●—●), but replaced by distilled water in the third cuvet (phosphohydroxypyruvate control, □—□). Reaction was initiated at zero time by the addition of 0.03 mg of phosphoglycerate dehydrogenase, to give a final volume of 2.25 ml, and followed spectrophotometrically at 340 mμ. Measurements were made against a blank containing all components except NADPH. At 6 minutes (1st arrow), each cuvet received 0.1 ml of *p*-mercuribenzoate, 10 μmoles, to inactivate phosphoglycerate dehydrogenase, followed by 0.2 ml of a solution containing 100 μmoles of magnesium sulfate and 10 μmoles of glucose-6-phosphate. At 9 minutes (2nd arrow), 0.1 ml of glucose-6-phosphate dehydrogenase was added to the phosphohydroxypyruvate control, □—□, and to one of the complete systems, △—△. To the second complete system was added 0.1 ml of distilled water (glucose-6-phosphate dehydrogenase control, ○—○). Total NADPH calculated from absorbance measurements and its molar extinction coefficient.

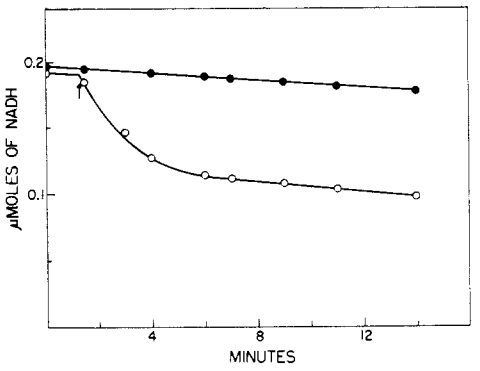

FIGURE 2: The identification of phosphoglycerate as the product of phosphohydroxypyruvate reduction with NADPH and phosphoglycerate dehydrogenase by a coupled assay with phosphoglycerate kinase and triose phosphate dehydrogenase. The enzymatic preparation of the two solutions assayed here and the assay conditions are described under Materials and Methods. The amount of phosphoglycerate in the aliquot of the complete system was calculated from the amount of NADH oxidized. O—O, complete system containing phosphohydroxypyruvate and NADPH; ●—●, NADPH blank used as a control for the nonspecific loss of NADH. Enzymes of coupled system added to cuvets at time indicated by arrow.

μmoles; GSH, 5 μmoles; 0.25 μmole of coenzyme; and 3.89 μmoles of phosphohydroxypyruvate in a total volume of 2.25 ml. The reaction was initiated by the addition of enzyme. Under these conditions, the rate of oxidation of NADPH was 140% of that observed with NADH. When the phosphohydroxypyruvate level was reduced to 0.1 μmole, NADPH was oxidized at less than 5% of the rate of oxidation of NADH.

The low reactivity observed with $NADP^+$ prompted further investigation of the reaction. Using a system containing glycylglycine, *p*H 7.4, 250 μmoles; EDTA, 25 μmoles; GSH, 5 μmoles; NADPH, 0.198 μmole; and 0.03 mg of protein in a total volume of 2.35 ml, it was shown that the addition of 0.10 μmole of phosphohydroxypyruvate resulted in the oxidation of 0.10 μmole of NADPH as determined spectrophotometrically. As additional proof that the triphosphopyridine nucleotides were reactive in this system, it was deemed necessary to identify $NADP^+$ and phosphoglycerate as the products of the reduction of phosphohydroxypyruvate in the presence of phosphoglycerate dehydrogenase and NADPH. These experiments are outlined in the following paragraphs.

Glucose-6-phosphate dehydrogenase was employed to measure the $NADP^+$ formed in the phosphoglycerate dehydrogenase system (Figure 1). In the complete system containing phosphohydroxypyruvate, phosphoglycerate dehydrogenase, and NADPH, essentially all of the NADPH was oxidized within 6 minutes. No reaction was observed in the absence of phosphohydroxypyruvate. $NADP^+$ was established as the product of this reaction since it served as a coenzyme for the glucose-6-phosphate dehydrogenase system. No reaction was observed in the absence of added glucose-6-phosphate dehydrogenase. Control experiments showed that the initial rate of oxidation of the enzymatically produced $NADP^+$ was identical to that of a system containing authentic $NADP^+$. The amount of NADPH produced (0.171 μmole) after 50 minutes by the oxidation of glucose-6-phosphate correlates well with the 0.188 μmole of $NADP^+$ produced by the reduction of phosphohydroxypyruvate. NAD^+ could not replace $NADP^+$ in the oxidation of glucose-6-phosphate.

A coupled assay of phosphoglycerate kinase and triosephosphate dehydrogenase was used to measure the phosphoglycerate produced by the enzymatic reduction of phosphohydroxypyruvate with NADPH. The details are described under Materials and Methods. In the complete system containing phosphohydroxypyruvate, phosphoglycerate dehydrogenase, and NADPH, 0.166 μmole of NADPH was oxidized. An aliquot containing 43.5% of the reaction products was assayed for phosphoglycerate (Figure 2). The NADH oxidized by triosephosphate dehydrogenase is equivalent to the amount of phosphoglycerate in the aliquot. The oxidation of 0.166 μmole of NADPH by phosphohydroxypyruvate resulted in the formation of 0.166 μmole of phosphoglycerate. A control system showed that phosphoglycerate production was dependent upon the presence of phosphohydroxypyruvate.

The substrate specificity of the phosphoglycerate dehydrogenase preparation was investigated. In addition, since Umbarger and Umbarger (1962) and Pizer (1963) have shown that bacterial phosphoglycerate dehydrogenase is inhibited by L-serine, the effect of this compound, as well as other metabolites, on the activity of the chicken liver enzyme was examined. In this series of experiments the activities of the enzyme with phosphoglycerate alone, metabolite alone, and phosphoglycerate plus metabolite were compared. The 2.25-ml incubation system contained Tris-chloride buffer, *p*H 8.0, 500 μmoles; hydrazine acetate, *p*H 8.0, 400 μmoles; NAD^+, 1.5 μmoles; GSH, 5 μmoles; enzyme; D-3-phosphoglycerate, 5 μmoles (omitted from metabolite control); and metabolite, 5 μmoles (omitted from phosphoglycerate control). The detection of activity with the metabolite alone served as a determination of the substrate specificity of the enzyme preparation. The activity observed with phosphoglycerate alone was equal to an absorbance change of 0.02/min at 340 mμ. Under these conditions, it was observed that none of the following compounds either affected the activity of phosphoglycerate dehydrogenase or reacted as substrates in the system: L-serine, *O*-phospho-L-serine, D-glycerate, hydroxypyruvate, pyruvate, glycolate, glyoxylate, formate, alanine, glycine, DL-3-phosphoglyceraldehyde, DL-glyceraldehyde, glycolaldehyde, formaldehyde, citrate, or glucose-6-phosphate. L-Malate had 4% and D-2-phosphoglycerate had 40% of the activity of D-3-phosphoglycerate. The latter observation could be accounted for fully by the presence of D-3-phosphoglycerate in the preparation of D-2-

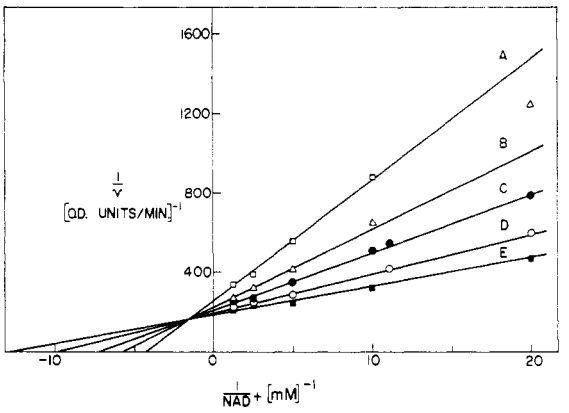

FIGURE 3: Double-reciprocal plots of initial velocity versus NAD^+ concentration at various constant levels of phosphoglycerate. The concentrations of phosphoglycerate used were: (A) 5×10^{-4} M; (B) 1×10^{-3} M; (C) 2×10^{-3} M; (D) 4×10^{-3} M; (E) 8×10^{-3} M. Other experimental conditions are described in the text.

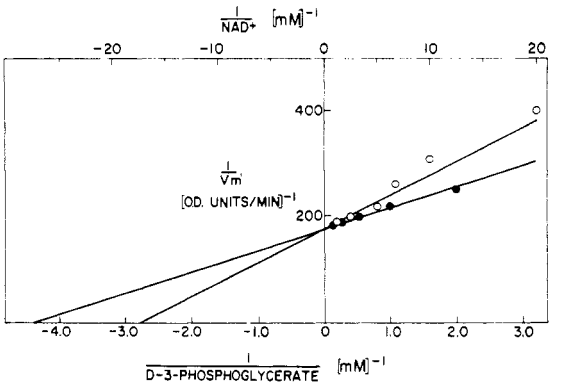

FIGURE 4: Double-reciprocal plot of the apparent maximum velocities versus phosphoglycerate (●—●) and NAD^+ (○—○) concentrations. The apparent maximum velocities were determined from the intercept of the double-reciprocal plots of initial velocity versus substrate concentrations. Experimental conditions are described in the text.

phosphoglycerate used. The enzyme had between 5 and 35% activity with L-lactate when the enzyme was assayed in the standard assay system with L-lactate replacing D-3-phosphoglycerate. The enzyme preparation used for the kinetic studies had 6% activity with L-lactate.

Evaluation of the Kinetic Constants of D-3-Phosphoglycerate Dehydrogenase. The initial velocities presented in Figure 3 were obtained by performing the reaction at varying concentrations of NAD^+ at several fixed concentrations of phosphoglycerate. The results show that phosphoglycerate dehydrogenase is similar to a number of other dehydrogenases in that the apparent Michaelis constant, K_m', for NAD^+ is dependent on the concentration of the oxidized substrate. The data of Figure 3 can be represented by the equation:

$$v = \frac{V_f}{1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{ab}}{[A][B]}} \qquad (1)$$

where v is the initial velocity, V_f is the maximum velocity in the forward direction, K_a and [A] refer, respectively, to the Michaelis constant and the concentration of NAD^+, K_b and [B] refer, respectively, to the Michaelis constant and concentration of phosphoglycerate, and K_{ab} is a complex constant as described by Alberty (1953). He has shown that rate equation (1) may represent any of the three following types of enzymatic reaction. These are (a) a random-sequence mechanism in which the interconversion of the ternary intermediates is rate limiting and the binding of the first substrate influences the binding of the second substrate; (b) a compulsory order of substrate binding such that the second substrate binds only to the enzyme–first substrate complex to form the ternary complex; and (c) the Theorell-Chance mechanism (1951) in which there is a compulsory order of substrate binding, but no kinetically significant ternary complex.

The enzymatic mechanism of most dehydrogenases so far examined has been shown to be type (b) with the pyridine nucleotide as the first substrate. The evidence accumulated for such a mechanism for lactate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase, glutamate dehydrogenase, and a number of other dehydrogenases has recently been compiled by Shifrin and Kaplan (1960) and by Alberty (1962). The evidence presented later in this paper, concerning the protection by pyridine nucleotides of phosphoglycerate dehydrogenase from inhibition by 3-bromopyruvate and NEM, supports the hypothesis that pyridine nucleotides are bound to the enzyme in the absence of phosphoglycerate or phosphohydroxypyruvate. Although no complete evidence has been presented, the kinetics of phosphoglycerate dehydrogenase will be interpreted mainly in terms of such a mechanism:

$$E + A \leftrightharpoons EA,\ EA + B \leftrightharpoons EAB \leftrightharpoons ECD \leftrightharpoons ED + C,\ ED \leftrightharpoons E + D$$

A, B, C, D, E, represent NAD^+, phosphoglycerate, phosphohydroxypyruvate, NADH, and enzyme, respectively. If the foregoing mechanism is correct, then the dissociation constant of NAD^+ is equal to the negative NAD^+ concentration at the point of intersection of the plots in Figure 3 (Frieden, 1957). Thus the dissociation constant for NAD^+ is equal to 7.0×10^{-4} M. Figure 4 shows the secondary plot of the data of Figure 3. From this plot the value of the Michaelis constant for phosphoglycerate (K_b) is calculated to be 2.5×10^{-4} M. The experimental values of Figure 3 were replotted to give the apparent maximum velocities (V_m') at fixed NAD^+ concentrations. The Michaelis constant for

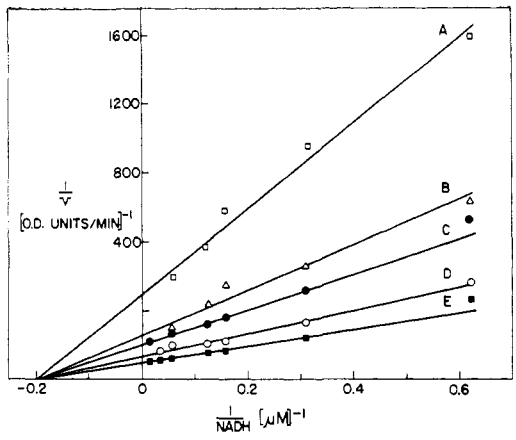

FIGURE 5: Double-reciprocal plots of initial velocity versus NADH concentration at various constant levels of phosphohydroxypyruvate. The concentrations of phosphohydroxypyruvate used were: (A) 1.25×10^{-6} M; (B) 2.50×10^{-6} M; (C) 3.75×10^{-6} M; (D) 6.75×10^{-6} M; (E) 12.50×10^{-6} M. Other experimental conditions are described in the text.

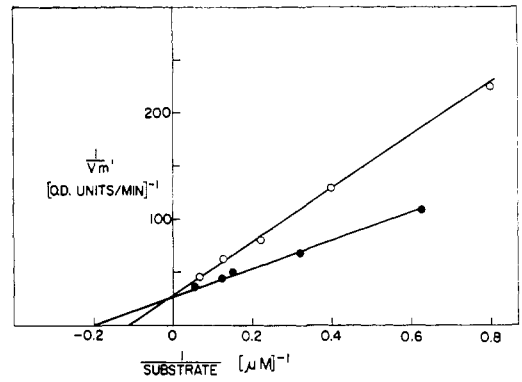

FIGURE 6: Double-reciprocal plot of the apparent maximum velocities versus phosphohydroxypyruvate (O—O) and NADH (●—●) concentrations. The apparent maximum velocities were determined from the intercepts of the double-reciprocal plots of initial velocity versus substrate concentration. Experimental conditions are described in the text.

NAD^+ (K_a) is calculated to be 6.0×10^{-5} M from the secondary plot of these V_m' values (Figure 4). The value of K_{ab} has been shown by Frieden (1957) to be equal to the product of K_b times the dissociation constant of A. This constant thus has the value of 1.6×10^{-7} moles^2/liter^2.

The data obtained for the reduction of phosphohydroxypyruvate are presented in Figure 5. These results can be represented by the equation (Alberty, 1953):

$$v = \frac{V_r}{\left[1 + \frac{K_c}{[C]}\right]\left[1 + \frac{K_d}{[D]}\right]} \qquad (2)$$

where V_r is the maximum velocity in the reverse direction, K_c and [C] refer, respectively, to the Michaelis constant and the concentration of phosphohydroxypyruvate, and K_d and [D] are the Michaelis constant and concentration of NADH, respectively. The interpretation of the data in the reverse reaction is analogous to that of the forward reaction, however, in this case $K_{cd} = K_c \times K_d$, and equation (1) reduces to equation (2). Using the same mechanistic interpretation for the reverse as for the forward reaction, the data (Figure 5) show that the Michaelis constant for NADH is equal to the dissociation constant of the NADH-enzyme complex (5×10^{-6} M). The Michaelis constant for phosphohydroxypyruvate (K_c), calculated from the secondary plot shown in Figure 6, is equal to 1×10^{-5} M.

Florini and Vestling (1957) have shown that the ordinates of the points of intersection ($1/v'$) of the double-reciprocal plots is given by:

$$\frac{1}{v'} = \frac{K_{ab} - K_a K_b}{K_{ab} V_f}$$

These authors pointed out that since the calculated value of this term involved all four kinetic constants in a given reaction direction, it may be compared to the graphically obtained values as an index of the accuracy of the data and the self-consistency of an experiment. Using this criterion, the kinetically determined constants were found to be in good agreement with the experimental data.

The equilibrium constant for the reaction can be calculated from the kinetic constants. Alberty (1953) has shown from the Haldane relationship for mechanism (b) that the equilibrium constant is given by:

$$K_{eq} = \frac{V_f K_c K_d}{V_r K_{ab}} [H^+]$$

The kinetic constants are summarized in Table II.

TABLE II: Kinetic Constants for Phosphoglycerate Dehydrogenase at pH 8.0.

Constant	Substrate	Experimental Value (M)
Michaelis	NAD^+	6.0×10^{-5}
Michaelis	Phosphoglycerate	2.5×10^{-4}
Michaelis	NADH	5.0×10^{-6}
Michaelis	Phosphohydroxypyruvate	1.0×10^{-5}
Dissociation	NAD^+	7.0×10^{-4}
Dissociation	NADH	5.0×10^{-6}
Equilibrium	Phosphoglycerate oxidation	1×10^{-12}

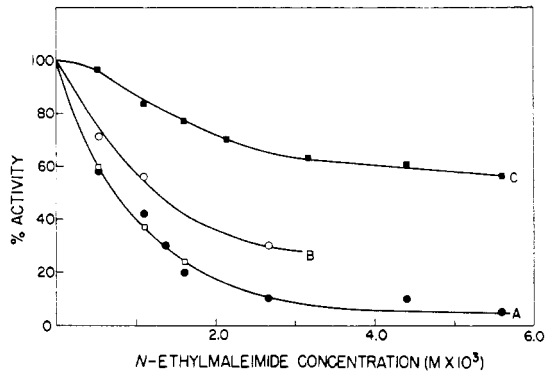

FIGURE 7: Protection of phosphoglycerate dehydrogenase by substrates from inhibition by NEM. The activity of the enzyme was determined by the linear production of NADH over a 5-minute period measured spectrophotometrically at 1-minute intervals at 340 mμ. All assay systems contained Tris-chloride buffer, *p*H 9.0, 500 μmoles; hydrazine acetate, *p*H 9.0, 400 μmoles; EDTA, 25 μmoles; 6.1×10^{-3} mg protein. Curve A (●—●), the variation of activity of phosphoglycerate dehydrogenase with NEM concentration. At 1 minute a given amount of NEM was added to the assay system to give a total volume of 1.9 ml. At 2 minutes 0.35 ml of a solution of 25 μmoles of phosphoglycerate and 1.5 μmoles of NAD^+ was added. Optical density was recorded between 3 and 8 minutes. Curve B (○—○), the protection by preincubation with NAD^+. At zero time the assay system was mixed with 1.5 μmoles of NAD^+. At 1 minute NEM was added to give a total volume of 1.9 ml. At 2 minutes 25 μmoles of phosphoglycerate was added to give a final volume of 2.25 ml. Optical density was recorded between 3 and 8 minutes. (□—□), the effect of preincubation with phosphoglycerate. The data were obtained by a procedure similar to that of curve B with the exception that the order of addition of phosphoglycerate with NAD^+ was reversed. Curve C (■—■), the protection by preincubation with NADH. At zero time the assay system was mixed with 2.03×10^{-2} μmoles of NADH. At 1 minute NEM was added to give a final volume of 1.9 ml. At 2 minutes 0.35 ml of a solution of 25 μmoles of phosphoglycerate and 1.5 μmoles of NAD^+ was added. Optical density was recorded between 3 and 8 minutes. All inhibitor concentrations reported refer to the initial incubation (1.9 ml).

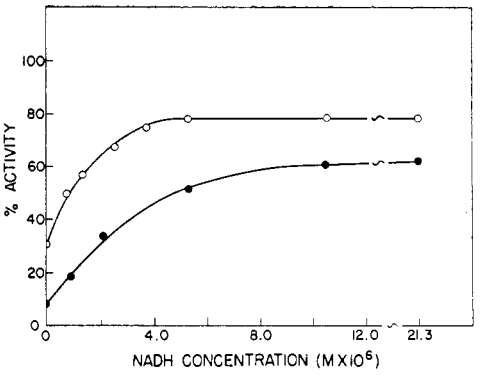

FIGURE 8: Variation of the inhibition of phosphoglycerate dehydrogenase by NEM with NADH concentration. The experimental conditions are described in the legend of Figure 7. NEM concentrations used were 1.57 mM (○—○) and 4.2 mM (●—●).

It has recently been shown that a number of enzyme systems are activated or inhibited by purine nucleotides. The possible effect of nucleotides on the activity of phosphoglycerate dehydrogenase was examined in a system identical to that used for the determination of the kinetic constants, using a concentration of 2×10^{-3} M phosphoglycerate and 2×10^{-4} M NAD^+. In those assays where magnesium chloride was added, EDTA was omitted. It was found that none of the following nucleotides had any significant effect on the activity of the enzyme in the presence or absence of 1×10^{-2} M magnesium: ATP (5×10^{-6}–5×10^{-4} M); ADP (1×10^{-6}–1×10^{-3} M); AMP (5×10^{-3} M).

Reaction of Phosphoglycerate Dehydrogenase with Analogs of NAD^+. The apparent Michaelis constants (K_m') and the apparent maximum velocities (V_m') were determined for four analogs of NAD^+ and are given in Table III.

TABLE III: Apparent Michaelis Constants and Apparent Maximum Velocities of Analogs of NAD^+.[a]

Nucleotide	K_m' (M)	V_m' [b] (%)
NAD^+	1.4×10^{-4}	100
3-Acetylpyridine-NAD^+	9.0×10^{-5}	540
3-Acetylpyridine-deamino-NAD^+	3.0×10^{-4}	105
3-Pyridinealdehyde-NAD^+	2.0×10^{-3}	95
Deamino-NAD^+	7.0×10^{-3}	210

[a] Results were determined from double-reciprocal plots obtained at 2×10^{-3} M phosphoglycerate under conditions described in the text. [b] Expressed as per cent of the apparent maximum velocity of NAD^+ obtained under identical conditions.

Inhibition of Phosphoglycerate Dehydrogenase with NEM. NEM reacts with thiol groups to produce thioethers. The specificity of this reaction is not high and derivatization of other functional groups of proteins by NEM would be expected. Phosphoglycerate dehydrogenase is inhibited by this reagent. Curve A in Figure 7 shows the variation of enzymatic activity with NEM concentration. (In Figures 7, 8, and 9, the activities of the enzyme are expressed as percentage

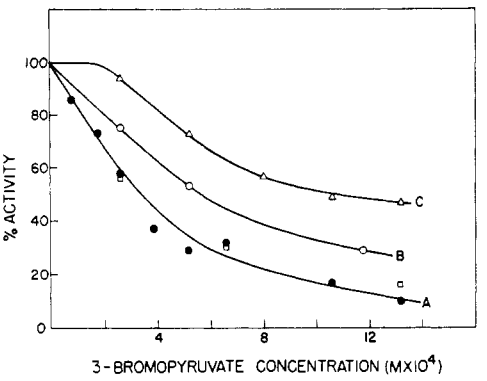

FIGURE 9: Protection of phosphoglycerate dehydrogenase by substrates from inhibition by 3-bromopyruvate. Curve A (●—●), the variation of activity of enzyme with 3-bromopyruvate concentration. Curve B (○—○), the protection by preincubation with NAD^+. Curve C (△—△), the protection by preincubation with NADH. (□—□), the effect of preincubation with phosphoglycerate. The experimental conditions are those described in the legend of Figure 7 with the exception that 3-bromopyruvate replaced NEM.

of the activity of the enzyme treated in an identical manner in the absence of inhibitor.) An increase in the time of preincubation of the enzyme with NEM to 7 minutes did not affect the final extent of inhibition. A concentration of 6.8×10^{-4} M NEM in the initial incubation system resulted in a 50% inhibition of the enzyme. The data represented by curve C of Figure 7 show the partial protection of the enzyme from NEM inhibition by preincubation with 2.03×10^{-2} μmole of NADH. It was shown in control experiments that this level of NADH did not significantly alter the initial rates of oxidation of phosphoglycerate in the presence of the uninhibited enzyme. The variation of NEM inhibition with NADH concentration is shown in Figure 8 at two concentrations of NEM. At low levels of NADH increasing protection was obtained by increasing the NADH concentration. The final level of protection obtained, at the concentrations of NEM used, was less than 100% and decreased with increasing NEM concentrations. The data represented by curve B of Figure 7 demonstrate that preincubation of the enzyme with NAD^+ partially protects the enzyme from NEM inhibition, but the amount of protection is less than that obtained with NADH. These results indicate that NAD^+ or NADH may bind to the enzyme in the absence of phosphoglycerate or phosphohydroxypyruvate. The data represented by the open squares in Figure 7 show that preincubation of the enzyme with phosphoglycerate does not protect the enzyme from inhibition by NEM.

Inhibition of Phosphoglycerate Dehydrogenase by Alkylating Agents. The inhibition of phosphoglycerate dehydrogenase by iodoacetate was investigated. The enzyme was inhibited 50% by approximately 0.04 M iodoacetate at *p*H 8.0. However, since the compound has a significant absorption at 340 mμ at such high concentrations, no detailed studies were made with this reagent.

Recently, Heinrikson *et al.* (1964) have reported the use of 3-bromopyruvate as an alkylating agent for ribonuclease. Because of the structural similarities of this compound to phosphohydroxypyruvate, it was considered that 3-bromopyruvate might be a more specific inhibitor of the enzyme. Curve A of Figure 9 shows the variation of inhibition of phosphoglycerate dehydrogenase with increasing concentrations of 3-bromopyruvate. NAD^+ (curve B) and NADH (curve C) protected the enzyme from inhibition by 3-bromopyruvate. Preincubation with phosphoglycerate (open squares) did not protect the enzyme from inhibition. The patterns of inhibition and of protection by substrates are similar for both 3-bromopyruvate and NEM.

Inhibition of Phosphoglycerate Dehydrogenase by Organic Mercurials. The enzyme was inhibited by *p*-mercuriphenylsulfonate and by *p*-mercuribenzoate. Using the standard assay system, 6.1×10^{-3} mg of enzyme was inhibited 50% by 5.0×10^{-4} μmole of either of the organic mercurials. The percentage inhibition varied linearly with the concentration of *p*-mercuriphenylsulfonate between 10 and 90% inhibition. Preincubation with NADH did not protect the enzyme from inhibition by *p*-mercuribenzoate under these conditions.

Discussion

Metabolic reactions in which D-3-phosphoglycerate participates include those catalyzed by phosphoglycerate kinase, 2,3-phosphoglycerate mutase, and phosphoglycerate dehydrogenase. The properties of the first two enzymes from rabbit muscle have been investigated by Rao and Oesper (1961) and Grisolia (1962), respectively. The equilibria for all three reactions utilizing phosphoglycerate lie in favor of its formation and the Michaelis constants for phosphoglycerate for the three enzymes are all of the same order of magnitude (2×10^{-4}–5×10^{-3} M). If one may correlate information from two different animal tissues, then it would appear that the intracellular concentration of phosphoglycerate may not be an important criterion of its utilization.

Phosphoglycerate dehydrogenase catalyzes the first reaction in one of the major pathways for serine biosynthesis. The two other enzymes in this sequence are phosphohydroxypyruvate–glutamate transaminase and phosphoserine phosphatase. Phosphoglycerate dehydrogenase from *S. typhimurium* (Umbarger and Umbarger, 1962) and *E. coli* (Pizer, 1963) has been shown to be sensitive to feedback inhibition by the end product, L-serine. It has been suggested by Neuhaus and Byrne (1958) and by Borkenhagen and Kennedy (1958) that the observed inhibition of phosphoserine phosphatase by L-serine is an important regulatory mechanism of serine biosynthesis in animal tissues. The inhibition of phosphoserine phosphatase from

E. coli by L-serine has been reported (Pizer, 1963). No inhibition by L-serine or any of a number of other metabolites was observed in these studies with the animal phosphoglycerate dehydrogenase. Gerhart and Pardee (1962) have shown that aspartate transcarbamylase is inhibited by cytidine triphosphate, but that it is possible to destroy the inhibition completely while leaving the enzyme fully active. Therefore, it is possible that the lack of an effect by L-serine or other metabolites on animal phosphoglycerate dehydrogenase may have been owing to the methods used in the purification of enzyme. However, Pizer (1964) has recently shown with cultured human cells that L-serine inhibits phosphoserine phosphatase, but he observed no effect with this compound on the activity of phosphoglycerate dehydrogenase. It would appear, therefore, that the bacterial and animal phosphoglycerate dehydrogenases differ in this respect.

Phosphoglycerate dehydrogenase was shown to be inhibited by NEM, 3-bromopyruvate, and *p*-mercuribenzoate. NADH and NAD^+ partially protected the enzyme from inhibition by the first two reagents, but no protection from the last inhibitor was observed. These facts may be interpreted as a differential derivatization of protein groups or as differences in the overall reactivity of the reagents with the protein. These results show that NADH and NAD^+ are bound to the enzyme in the absence of phosphoglycerate and phosphohydroxypyruvate, but do not prove absolutely that this binding occurs at the active site. However, the fact that the protection by NADH was in the same concentration range as the Michaelis and dissociation constants for NADH (Figure 8) lends support to this interpretation.

The greater initial reaction rates observed with 3-acetylpyridine-NAD^+, as compared to those of NAD^+, suggest that the former nucleotide would be very useful in a sensitive assay for phosphoglycerate dehydrogenase.

Acknowledgments

The authors are indebted to Dr. L. A. Fahien for helpful discussions and advice relating to the kinetic studies. The technical assistance of Miss Sandra Langhofer and Mr. Edward Kmiotek is gratefully acknowledged.

References

Alberty, R. A. (1953), *J. Am. Chem. Soc. 75*, 1928.
Alberty, R. A. (1962), *J. Am. Chem. Soc. 84*, 4375.
Ballou, C. E. (1960), *Biochem. Prepn. 7*, 66.
Borkenhagen, L. F., and Kennedy, E. P. (1958), *Biochim. Biophys. Acta 28*, 222.
Bucher, T. (1955), *Methods Enzymol. 1*, 415.
Cleland, W. W. (1964), *Biochemistry 3*, 480.
Cori, G. T., Slein, M. W., and Cori, C. F. (1948), *J. Biol. Chem. 173*, 605.
Dalziel, K. (1963), *J. Biol. Chem. 238*, 1538.
Evans, R. M., Fraser, J. B., and Owen, L. N. (1949), *J. Chem. Soc.*, 248.
Florini, J. R., and Vestling, C. S. (1957), *Biochim. Biophys. Acta 25*, 575.
Frieden, C. (1957), *J. Am. Chem. Soc. 79*, 1894.
Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.
Grisolia, S. (1962), *Methods Enzymol. 5*, 236.
Hanford, J., and Davies, D. D. (1958), *Nature 182*, 532.
Heinrikson, R. L., Crestfield, A. M., Stein, W. H., and Moore, S. (1964), *Proc. Intern. Congr. Biochem. 6th, New York*, 313.
Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.
Ichihara, A., and Greenberg, D. M. (1957), *J. Biol. Chem. 224*, 331.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Metzenberg, R. L., Marshall, M., and Cohen, P. P. (1960), *Biochem. Prepn. 7*, 23.
Neuhaus, F. C., and Byrne, W. L. (1958), *Biochim. Biophys. Acta 28*, 223.
Neuhaus, F. C., and Korkes, S. (1958), *Biochem. Prepn. 6*, 75.
Pizer, L. (1963), *J. Biol. Chem. 238*, 3934.
Pizer, L. (1964), *J. Biol. Chem. 239*, 4219.
Rao, D. R., and Oesper, P. (1961), *Biochem. J. 81*, 405.
Shifrin, S., and Kaplan, N. O. (1960), *Advan. Enzymol. 22*, 337.
Siegel, J. M., Montgomery, G. A., and Bock, R. M. (1959), *Arch. Biochem. Biophys. 82*, 288.
Sprinson, D. B., and Chargaff, E. (1946), *J. Biol. Chem. 164*, 417.
Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127.
Umbarger, H. E., and Umbarger, M. A. (1962), *Biochim. Biophys. Acta 62*, 193.
Warburg, O., and Christian, W. (1942), *Biochem. Z. 310*, 384.
Willis, J. E., and Sallach, H. J. (1962), *J. Biol. Chem. 237*, 910.
Willis, J. E., and Sallach, H. J. (1964), *Biochim. Biophys. Acta 81*, 39.
Winer, A. D., and Schwert, G. W. (1958), *J. Biol. Chem. 231*, 1065.