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D-3-PHOSPHOGLYCERATE DEHYDROGENASE. FURTHER STUDIES ON THE ENZYME ISOLATED FROM CHICKEN LIVER

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SUMMARY

1. Additional purification of D-3-phosphoglycerate dehydrogenase (D-3-phosphoglycerate:NAD oxidoreductase) from chicken liver has been achieved. By the criteria of ultracentrifugation, gel filtration on Bio-Gel P-200, and starch-gel electrophoresis, the protein preparation is of a high degree of purity.

2. Chicken-liver phosphoglycerate dehydrogenase has a molecular weight of approx. $2 \cdot 10^5$. The enzyme exhibits a multiplicity of enzymatically active zones on polyacrylamide-gel electrophoresis. The amino acid composition of the enzyme is reported.

INTRODUCTION

A previous communication from this laboratory[1] described the first extensive purification of D-3-phosphoglycerate dehydrogenase (D-3-phosphoglycerate:NAD oxidoreductase), the enzyme that catalyzes the formation of phosphohydroxypyruvate from D-3-phosphoglycerate. The enzyme from chicken liver is specific, in the range of physiological concentrations, for nicotinamide-adenine dinucleotides. Phosphoglycerate dehydrogenase has been demonstrated to be present in a wide variety of vertebrate tissues[2] and to be subject to metabolic adaptation in mammalian systems[3]. The enzyme is present in plants[4] and in extracts of *Salmonella typhimurium*[5], *Peptostreptococcus elsdenii*[6], and *Escherichia coli*[7]. The protein from this latter source has recently been crystallized and shown to be homogeneous in the ultracentrifuge[8]. Bacterial phosphoglycerate dehydrogenase, in contrast to the enzyme from animal sources, is markedly inhibited by the end product, L-serine[5,7]. The present communication describes the further purification of avian phosphoglycerate dehydrogenase and physicochemical studies with the enzyme.

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MATERIALS AND METHODS

Preparation of the column of Bio-Gel P-200 for gel filtration

A glass column (60 cm × 4 cm) was acid-cleaned and coated with silicone by the application of a solution of dichlorodimethylsilane (1% in benzene). Bio-Gel P-200 was suspended in 0.1 M potassium phosphate buffer (pH 6.1) containing $3 \cdot 10^{-3}$ M EDTA, and the fine gel particles were removed by decantation. The gel suspension was deaerated *in vacuo* at 25°, and then allowed to equilibrate at 4° before the chromatographic column was prepared. A 2-cm layer of Sephadex G-25 that had been equilibrated with the same phosphate buffer was placed on top of the column of gel. Immediately prior to the application of the protein, the gel was washed with at least 300 ml of 0.1 M potassium phosphate buffer (pH 6.1) containing $3 \cdot 10^{-3}$ M EDTA and $0.5 \cdot 10^{-3}$ M β-mercaptoethanol. The column was then washed with an additional volume of the same buffer with the exception that the β-mercaptoethanol was replaced by $3 \cdot 10^{-4}$ M dithiothreitol. The volume of the second washing of buffer was equal to the total volume of the column. The column was then ready for use.

Amino acid analyses

The amino acid composition of phosphoglycerate dehydrogenase was determined from a sample of the purified protein that had been exhaustively dialyzed against doubly distilled water. Samples of between 0.5 to 1.0 mg of lyophilized protein were subjected to hydrolysis for 22 and 44 h at 110° with 1 ml of 6 M HCl in evacuated, sealed, acid-washed pyrex tubes. Samples of the hydrolysates were analyzed with an automatic amino acid analyzer (Beckman Model 120B), according to the method described by MOORE, SPACKMAN AND STEIN[9,10]. The total methionine and the half cystine contents were estimated by the performic acid oxidation of the protein according to the procedure of MOORE[11]. No assays were performed to determine either the tryptophan content or the presence of free sulfhydryl groups in the native protein.

Electrophoretic studies

(a) Vertical starch-gel electrophoresis was performed at 4° as described by SMITHIES[12] using a gel composition of 12%. At the termination of the electrophoresis, each of the gels was bisected, and one half was stained for protein with Amido Black. The other half of the gel was used to localize phosphoglycerate dehydrogenase activity by an adaptation of the general method described for dehydrogenases by FINE AND COSTELLO[13]. The gel was incubated in the dark at 37° with a solution containing: Tris chloride buffer (pH 8.4), 6 mmoles; NAD^+, 22.5 μmoles; D-3-phosphoglycerate, 2.8 mmoles; phenazine methosulfate, 1.25 mg; and *p*-nitro blue tetrazolium, 30 mg; in a total volume of 15 ml. The reaction was terminated by washing the gel with a 7% solution of acetic acid.

(b) Polyacrylamide disc electrophoresis[14,15] was conducted at 10° with an apparatus designed by Dr. R. L. METZENBERG of this department. A continuous buffer system was used according to the principles outlined by HJERTEN, JERSTEDT AND TISELIUS[16]. The buffer in the gel and the anode compartment was 0.05 M potassium phosphate (pH 6.0) containing $1 \cdot 10^{-4}$ M EDTA. The buffer in the cathode

compartment was 0.01 M potassium phosphate (pH 6.0) containing $1 \cdot 10^{-4}$ M EDTA. The gels (5%) were polymerized in the absence of EDTA, catalyzed by *N,N,N',N'*-tetramethylethylenediamine (6.5 μl/100 ml) and ammonium persulfate (4 mg/ml). The gels were subjected to electrophoresis in the presence of EDTA for 3 h prior to the application of the protein under conditions such that the phosphate concentration of the gel remained constant (0.05 M). Protein was applied in 0.05 ml of a 4% glycerol solution in the same phosphate buffer as used in the cathode compartment by layering through the cathode compartment buffer onto the top of the gel. Gels were stained for either enzymatic activity or protein by the same methods as described for starch-gel electrophoresis.

Enzyme and protein determinations

The standard assay conditions, the unit of enzymatic activity, and specific activity for phosphoglycerate dehydrogenase have been defined previously[2]. Protein concentrations were measured either by the method of LOWRY *et al.*[17], with bovine serum albumin as the standard, or by that of WARBURG AND CHRISTIAN[18].

Substrates, enzymes and other chemicals

The potassium salt of D-3-phosphoglycerate was prepared from the barium salt (Sigma Chemical Co.) as described previously[19]. NAD^+, phenazine methosulfate, *p*-nitro blue tetrazolium and pyruvate kinase (EC 2.7.1.40) were purchased from Sigma Chemical Co. Lactate dehydrogenase (EC 1.1.1.27) (Nutritional Biochemical Corp.), Blue Dextran 2000 (Pharmacia), Bio-Gel P-200 (Bio-Rad Laboratories), Amido black 10B (Hartman–Leddon Co.), Starch-hydrolyzed (Connaught Medical Research Laboratories, Toronto, Canada) and dichlorodimethylsilane (Aldrich Chemical Co.), were commercial products. The chemicals used for the formation of polyacrylamide gels for disc electrophoresis (acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine) were the products of Eastman Organic Chemicals. Dithiothreitol was prepared as described previously[1]. Other compounds used in these studies were commercial preparations.

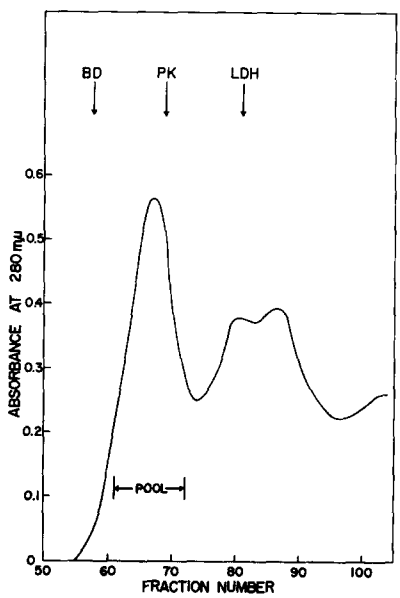
RESULTS

Purification of phosphoglycerate dehydrogenase from chicken liver by gel filtration

The initial procedures used for the purification of phosphoglycerate dehydrogenase from chicken liver were those described earlier[1]. The enzyme preparation used in these studies was the 0–62% $(NH_4)_2SO_4$ precipitate of Fraction H (see ref. 1). An aliquot of the $(NH_4)_2SO_4$ residue, containing approx. 400 000 units of enzymatic activity, was dissolved in 2 ml of 0.2 M potassium phosphate buffer (pH 7.2) containing 0.2 M NaCl, $3 \cdot 10^{-3}$ M EDTA, and $3 \cdot 10^{-4}$ M dithiothreitol. The solution was applied to a column of Bio-Gel P-200 (53 cm $\times$ 4 cm) that had been prepared as described in MATERIALS AND METHODS. The sample of protein was washed onto the column with three successive 2-ml additions of the same buffer with which the gel was equilibrated. The column of polyacrylamide gel was eluted with this same buffer at a flow rate of between 10 to 25 ml/h. The elution pattern of the chromatogram of Bio-Gel P-200 is shown in Fig. 1. The pooled fractions (61–72) of the eluate from the column of Bio-Gel containing phosphoglycerate dehydrogenase (45 ml) were

brought to 62% saturation by the careful addition of 18 g of $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation and could be stored at −15° with little loss in specific activity. The enzyme recovered in the pooled fractions contained an average of 75% of that applied to the column of Bio-Gel. This additional purification resulted in a 1.7-fold increase in specific activity.

The final purification procedure removes all traces of contaminating activities of lactate dehydrogenase and malate dehydrogenase (EC 1.1.1.37) that are present in the protein solution applied to the column. Lactate dehydrogenase from chicken liver has a molecular weight of $1.3 \cdot 10^5$ (see ref. 20), and would probably be eluted in the same fractions as lactate dehydrogenase from rabbit muscle under the conditions described for gel filtration on P-200 (Fig. 1). The purified enzyme had a specific activity of 25 000 and a $E_{280\ m\mu}/E_{260\ m\mu}$ ratio of 1.7.



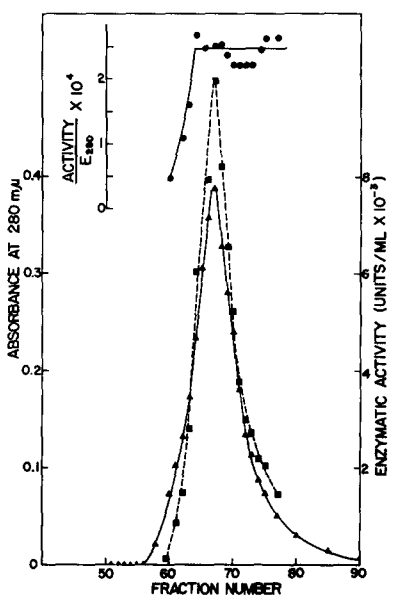


Fig. 1. Typical elution pattern of a Bio-Gel P-200 chromatogram used for the final purification step of chicken-liver phosphoglycerate dehydrogenase. The column (53 cm × 4 cm) was eluted with potassium phosphate buffer (pH 6.1) containing $3 \cdot 10^{-3}$ M EDTA and $3 \cdot 10^{-4}$ M dithiothreitol (flow rate, 15 ml/h). Each fraction contained 3.8 ml. The positions of the three arrows on the elution pattern mark the fractions in which Blue Dextran 2000 (BD, $2 \cdot 10^6$ mol. wt.), pyruvate kinase from rabbit muscle (PK, $2.37 \cdot 10^5$ mol. wt.), and lactate dehydrogenase from rabbit muscle (LDH, $1.32 \cdot 10^5$ mol. wt.) were eluted in separate control experiments. Fractions 61–72 were pooled and the protein precipitated as described in the text.

Fig. 2. Gel filtration of purified phosphoglycerate dehydrogenase on Bio-Gel P-200. The column size, elution buffer, flow rate, and volume of each fraction were exactly the same as described in the legend of Fig. 1. ■—■, enzymatic activity; ▲—▲, $A_{280\ m\mu}$; ●—●, ratio of enzymatic activity to $A_{280\ m\mu}$.

The purity and physicochemical characteristics of chicken-liver phosphoglycerate dehydrogenase

The following studies were performed to establish the purity of the enzyme preparation as well as some of the physicochemical properties of the protein.

Gel filtration

Purified phosphoglycerate dehydrogenase was chromatographed on a column of Bio-Gel P-200 under conditions that were identical to those described for the final purification step of the enzyme. The elution pattern of the chromatogram is shown in Fig. 2. The elution of the enzymatic activity coincides with that of the protein over a wide range of the chromatogram. Only in those fractions of the leading edge of the peak, in which the protein concentration is low, is the specific activity below the maximum level. The constancy of the ratio of the enzymatic activity to $A_{280\ m\mu}$, in all other fractions of the eluted protein, shows that phosphoglycerate dehydrogenase is free of contaminating proteins of low molecular weight. The void volume of the column used for this experiment as well as for the final purification step was determined from the elution of Blue Dextran 2000 (see Fig. 1). No proteins of high molecular weight, that would have eluted in the void volume of the column (Fraction 55), contaminate the enzyme preparation (Fig. 2).

Ultracentrifugation study

A velocity sedimentation study was performed with a sample of the purified avian phosphoglycerate dehydrogenase. An aliquot of the $(NH_4)_2SO_4$ residue, obtained from the final purification step, was dissolved in 0.8 ml of 0.1 M potassium phosphate buffer (pH 6.0) containing $3 \cdot 10^{-3}$ M EDTA and $0.5 \cdot 10^{-3}$ M β-mercaptoethanol. The resulting solution was dialyzed against the same buffer for 45 min with one change of buffer, and then centrifuged at 12 000 $\times$ g for 10 min. (Under these conditions, the NH_4^+ concentration was shown, by an assay with Nessler's reagent, to decrease to less than $1 \cdot 10^{-3}$ M.) The supernatant fluid, containing 4.5 mg of protein per ml, was centrifuged in a Spinco Model E analytical ultracentrifuge at 3.97°. The sedimentation velocity patterns are shown in Fig. 3. The symmetrical Schlieren

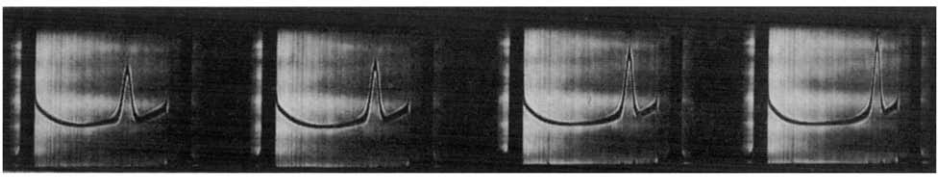


Fig. 3. Schlieren patterns of purified phosphoglycerate dehydrogenase obtained in the Spinco Model E ultracentrifuge at 3.97°. Photographs from right to left were taken at 24, 32, 40, and 48 min after reaching the final speed of 59 780 rev./min. The direction of sedimentation is from right to left. Prior to centrifugation the enzyme was dialyzed *vs.* 0.1 M potassium phosphate buffer (pH 6.0) containing $3 \cdot 10^{-3}$ M EDTA and $0.5 \cdot 10^{-3}$ M β-mercaptoethanol.

pattern observed is indicative of a protein of a high degree of homogeneity. The presence of a very slight trace of material, sedimenting faster than the main peak, is observed after centrifugation for 48 min. This material may be identical to one of the minor, enzymatically active, components identified by disc electrophoresis in this study. The $s_{20,w}$, calculated by the standard procedure of SVEDBERG AND PEDERSEN[21], is 7.3 S at a protein concentration of 4.5 mg/ml. Assays before and after ultracentrifugation established that no loss of enzymic activity occurred over the duration of the experiment.

Electrophoretic studies

The relative homogeneity of purified phosphoglycerate dehydrogenase was

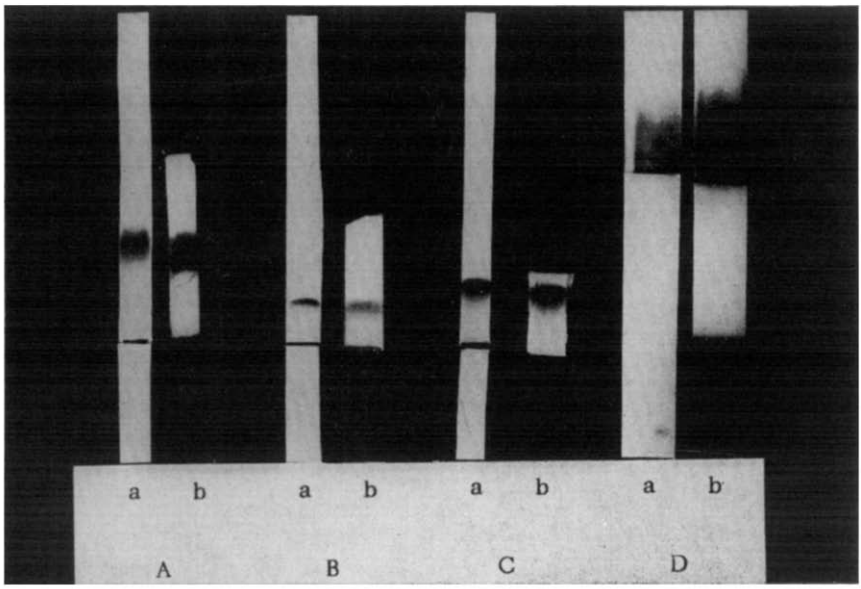


Fig. 4. Vertical starch-gel electrophoretic studies of chicken-liver phosphoglycerate dehydrogenase. A. In 0.02 M potassium acetate buffer (pH 4.6) containing $1 \cdot 10^{-3}$ M EDTA. B. In 0.01 M potassium phosphate buffer (pH 6.5) containing $1 \cdot 10^{-3}$ M EDTA. C. In the sodium citrate–sodium phosphate buffer (pH 7.0) described by FINE AND COSTELLO[18]. D. In 0.025 M sodium borate buffer (pH 8.6) containing $1 \cdot 10^{-3}$ M EDTA. Gels a and b of each experiment were stained for protein and enzymatic activity, respectively. The current (mA/cm^2) and the time (h) for each electrophoresis were, respectively, A, 2.3, 20; B, 2.9, 12; C, 2.9, 27; D, 2.3, 22. The direction of migration (bottom to top) is towards the cathode.

established by electrophoresis on starch and polyacrylamide gels. The solutions of enzyme used in these experiments were prepared by the same procedure as that described for the ultracentrifugation study. Other experimental details are described under MATERIALS AND METHODS. Phosphoglycerate dehydrogenase migrated as a single, enzymatically active, component in starch gel at pH's 4.6, 6.5 or 7.0 (Figs. 4A, B and C). At pH 8.6, in sodium borate buffer, the enzyme is apparently electrophoretically heterogeneous (Fig. 4D). The major fraction of the enzyme is basic at this pH and migrates as a very broad band, but a trace of enzymically active protein migrates towards the anode. No evidence was found by starch-gel electrophoresis of any non-enzymic impurity in the phosphoglycerate dehydrogenase preparation. Phosphoglycerate dehydrogenase migrated as more than one component on polyacrylamide disc electrophoresis at pH 6.0 (Fig. 5). A band of enzymically active protein that migrated slightly faster than the major band of enzyme was clearly detected even at low protein concentration. In addition, two other traces of protein, that migrated at approx. 50 and 200% of the rate of the major fraction, gave a very slight test for enzymic activity upon long incubation. These two components, however, constitute a very minute fraction of the total protein. Five bands of enzymically active protein were also obtained by disc electrophoresis of phosphoglycerate dehydrogenase in sodium cacodylate buffer (pH 6.4). CANN[22] has pointed out that multiplicity of electrophoretic zones due to buffer macromolecular interaction may account for the apparent electrophoretic heterogeneity of some proteins.

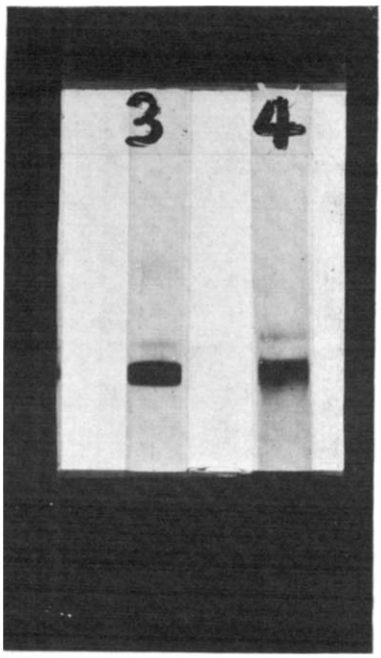


Fig. 5. Polyacrylamide disc electrophoresis of phosphoglycerate dehydrogenase at pH 6.0. Experimental details are described under MATERIALS AND METHODS. The gels were stained for protein (No. 3) and enzyme (No. 4). The direction of migration (bottom to top) is towards the cathode.

The apparent electrophoretic heterogeneity of liver alcohol dehydrogenase was demonstrated by MCKINLEY-MCKEE AND MOSS[23] to result from differential binding between the native enzyme and nicotinamide–adenine dinucleotides. Additional information is required to interpret the observed heterogeneity of phosphoglycerate dehydrogenase obtained by polyacrylamide disc electrophoresis.

Molecular weight of phosphoglycerate dehydrogenase

An estimate of the molecular weight of phosphoglycerate dehydrogenase was made from the results of the gel-filtration and sedimentation studies. The column used for both the final purification step and the gel filtration of the purified enzyme was calibrated in separate control experiments by the use of Blue Dextran and of proteins of known molecular weights (see Fig. 1). There was no difference between the elution volumes of phosphoglycerate dehydrogenase in the chromatograms used for either the final purification (Fig. 1) or the gel filtration of the purified enzyme (Fig. 2). Chicken-liver phosphoglycerate dehydrogenase was eluted in the same fractions as pyruvate kinase from rabbit muscle (mol. wt. $2.37 \cdot 10^5$; see ref. 24) on the column of Bio-Gel, 34 ml after the void volume. A clear separation was obtained between chicken-liver phosphoglycerate dehydrogenase and lactate dehydrogenase from rabbit muscle (mol. wt. $1.32 \cdot 10^5$; see ref. 25). The molecular weight of phosphoglycerate dehydrogenase from chicken liver, as determined by the criterion of cochromatography with pyruvate kinase, is approx. $2.3 \cdot 10^5$. There is a relatively good agreement between this value and that obtained by a comparison of the sedimentation coefficient of phosphoglycerate dehydrogenase determined in this study with the coefficients of proteins of known molecular weights.

TABLE I

AMINO ACID COMPOSITION OF CHICKEN-LIVER PHOSPHOGLYCERATE DEHYDROGENASE

The method of assay and the corrections used for the destruction of the labile amino acids are described in the text. Total protein was determined prior to the hydrolysis of the protein by the method of LOWRY *et al.*[17].

Constituent	*Amino acid residues (g/100 g of protein)*
Lys	7.5
His	2.5
Arg	6.5
Asp	8.7
Thr	6.9
Ser	6.3
Glu	14.8
Pro	4.6
Gly	8.0
Ala	9.1
CyS	3.0
Val	7.7
Met	2.6
Ile	4.6
Leu	14.2
Tyr	1.4
Phe	2.6
Total	113.8

Amino acid analyses of chicken-liver phosphoglycerate dehydrogenase

The amino acid content of phosphoglycerate dehydrogenase is presented in Table I. The extent of destruction of labile amino acids was calculated by extrapolation to zero time of the values obtained after 22 and 44 h of hydrolysis. The values of serine, threonine and aspartic acid have been corrected for losses in 22 h of 7.5%, 4%, and 2%, respectively. A correction of 6% loss was applied to the total half-cystine content, that was determined as cysteic acid[11].

DISCUSSION

The purification procedure for phosphoglycerate dehydrogenase established by the present and earlier[1] studies results in a protein preparation which by a number of physicochemical criteria is of a high degree of purity. However, due to the lability of the enzyme and the length of time required to achieve maximal purification, the quantity of enzyme available for studies of this type is very limited. On the basis of gel filtration and ultracentrifugation studies, the molecular weight of chicken-liver phosphoglycerate dehydrogenase may be estimated to be approx. $2 \cdot 10^5$. Although avian phosphoglycerate dehydrogenase is markedly different from the bacterial enzyme in its reactivity towards the allosteric inhibitor serine, no gross difference has been established yet between the sedimentation characteristics of the chicken-liver enzyme ($s_{20,w} = 7.3$) and that of the enzyme from *E. coli* ($s_{20,w} = 7.9$) as determined by SUGIMOTO AND PIZER[8]. The electrophoretic migration patterns, in

conjunction with the relative binding capacities of the enzyme with ion-exchange celluloses, indicate that phosphoglycerate dehydrogenase has a high isoelectric point (> pH 8.6). This fact, together with the data obtained for the amino acid composition, suggests that a large number of the glutamate and aspartate residues in the native enzyme are present as their amide derivatives.

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