BBA 66058

GLUCOSE DEHYDROGENASE FROM PIG LIVER

I. ISOLATION AND PURIFICATION

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(Received September 26th, 1969)

SUMMARY

1. Glucose dehydrogenase has been isolated from pig liver and crystallized from an ammonium sulfate solution.

2. This enzyme is capable of using either NAD or NADP, with a preference for NAD. If NADP is used, the enzyme will catalyze reactions where either glucose 1-phosphate or glucose 6-phosphate is the substrate.

3. The enzyme has a molecular weight of 235 000 and Stokes radius of 4.02 mμ. It is an unusually compact enzyme for its molecular weight.

4. The pH of maximum activity is 9.9 at an ionic strength of 0.1 M. The temperature range of greatest activity is 30–35°.

INTRODUCTION

In 1931 HARRISON[1] reported the existence of an enzyme "...capable, in the presence of a suitable hydrogen acceptor, of bringing about the oxidation of glucose". He also reported an extraction of this enzyme from bovine liver. The purification procedures were modified by BRUNELLI AND WANINIO[2] and STRECKER AND KORKES[3]. In 1964 METZGER *et al.*[4] reported the purification by means of gel filtration of the rat liver extract.

The enzyme glucose dehydrogenase, systematic name, β-D-glucose:NAD(P) oxidoreductase, EC 1.1.1.47 (ref. 5), catalyzes the direct oxidation of glucose to δ-glucono-lactone[3] with the accompanying reduction of a coenzyme, either NAD or NADP (ref. 6).

METZGER *et al.*[12] reported the extraction, purification and some physical characteristics of pig liver glucose dehydrogenase in 1965. In that study he reported a higher pH optimum than for any of the other sources of glucose dehydrogenase.

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The differences in the physical characteristics of pig liver glucose dehydrogenase compared with other sources are what prompted this study.

EXPERIMENTAL

Chemicals

β-D-Glucose, NAD, NADP, glucose 6-phosphate, and glucose 1-phosphate were obtained in the purest grades from California Biochemical Company. Other reagents were purchased from Mallinckrodt and Fisher Chemical companies in reagent grade. Lyphogel was obtained from the Gelman Instrument Company and Sephadex from Pharmacia.

Assay procedure

The assay procedure was essentially that of Strecker[7]: 2.6 ml buffer, 0.1 ml NAD (1.5 mM), and 0.1 ml enzyme solution were added to two identical cuvettes. To the blank cuvette was added 0.2 ml deionized water and both cuvettes were incubated for 10 min in the chamber of a Beckman DU spectrophotometer connected to a constant temperature water bath and circulator set at 30 ± 0.1°. At the end of the incubation period, 0.2 ml glucose solution (1 M) was added to the sample cuvette. Since the formation of reduced NAD produces an increase in absorbance at 340 mμ, the reaction was followed with absorbance readings at 30 and 90 sec after the addition of the glucose. All activities were recorded as change in absorbance of 0.001 per min. These values were later adjusted to the more recent definition[5] of one unit of activity being that amount of protein causing the formation of 1 μmole of NAD(P)H per min. Protein concentration was determined by $A_{280\ m\mu}/A_{260\ m\mu}$ measurements after WARBURG AND CHRISTIAN[10].

Sephadex column preparation and calibration

Sephadex was swollen in water either by standing at room temperature for 3 days or by standing in a 90° water bath for 1 day. The supernatant was decanted and replaced by 0.1% sodium azide three times, and the column packed and eluted for several days to stabilize the bed.

For calibration, the eluent between the bed and the top of the column was removed; a 4.0-ml sample of solution was added slowly by means of a syringe, and the column allowed to elute 4.0 ml of eluent. The eluent was replaced on top of the bed and the siphon reattached. The elution tube was attached to an automatic fraction collector (Gelman Medical Electronics) equipped with a timer.

The protein concentration was monitored by 280-mμ absorption, and the volume at peak absorption was taken as V_e (elution volume).

Enzyme preparation

Pig liver fresh from the slaughterhouse was cut into 2-cm cubes and frozen. Amounts of approx. 400 g were thawed, mixed with 2 parts acetone and homogenized in a Waring blender for approx. 1 min. Five parts acetone were added to this suspension and solution stirred for about 30 min. The suspension was filtered by suction on a large Buchner funnel and the resulting cake homogenized in a blender with 2 parts acetone for a second minute. Again the suspension was mixed with 5 parts

acetone and filtered. The cake was sucked as dry as possible. All of the above steps were performed in a cold room at approx. 4°.

The acetone powder cake was removed to room temperature, broken up by hand on paper towels. The powder was suspended in 8 vol. distilled water and stirred for approx. 30 min at room temperature. The suspension was allowed to settle for about 10 min and the supernatant poured through 4 layers of cheesecloth. The solid material was extracted a second time with water and the extracts combined while crude precipitate was discarded.

The combined water extracts were cooled in the cold room and the pH adjusted to approx. 6 with 10% acetic acid. The solution was centrifuged and the precipitate discarded. Ammonium sulfate (28 g per 100 ml of solution) was slowly added with stirring and the mixture stirred for 30 min. The solution was allowed to stand overnight and the total volume centrifuged at approx. 8000 × *g* for 15 min. The precipitate was collected and dissolved in distilled water and the protein concentration adjusted to approx. 6.0 g/100 ml. The resulting suspension was then centrifuged and the insoluble residue discarded.

The pH of the supernatant was lowered to 5.5 with 10% acetic acid. A 25-ml aliquot was removed and ammonium sulfate added in 0.25-g amounts with subsequent centrifugation and testing of the supernatant for activity until no more than 5% of the original activity had been lost. At this point the concentration of ammonium sulfate in the bulk solution was adjusted to that concentration in the aliquot just before the 5% loss in activity. The protein contaminant was allowed to precipitate for 1 h in the cold room, the mixture centrifuged at 7000 × *g* for 15 min, and the precipitate discarded. The protein concentration was then adjusted to approx. 4.5 g/100 ml and 18 g ammonium sulfate per 100 ml of solution added. The solution was allowed to stand overnight in the cold room. The mixture was then centrifuged at 14 000 × *g* for 30 min, the supernatant discarded, and the precipitate dissolved in distilled water.

The enzyme solution was concentrated in Lyphogel to remove contaminant of mol. wt. less than 20 000. This solution was then added to a 2.5 cm × 45 cm column of Sephadex G-200 in batches of not greater than 30 ml and eluted with distilled water (pH 7.0) containing approx. 0.1% sodium azide. 10-min fractions of approx. 2 ml were collected by means of a Gelman Medical Electronics fraction collector equipped with a timer. The enzyme was located by the assay method. The middle cut of fractions was combined and concentrated by Lyphogel and the resultant solution of not more than 10 mg/ml protein was run through a second column, this of Sephadex G-150 superfine. The fractions of approx. 2 ml were collected at 20-min intervals. Both columns were maintained at room temperature with no appreciable cost in enzyme activity.

Crystallization

The most active fractions from the second column run of several preparations were combined and concentrated with Lyphogel. The resulting solution was made 29% saturated with ammonium sulfate (200 mg/ml) and allowed to stand overnight in the cold room (4°). The precipitate was discarded and the solution made 36% saturated (250 mg/ml) by the addition of ammonium sulfate. The precipitate resulting from an overnight precipitation in the cold room was dissolved in deionized water

and ammonium sulfate added to 23% saturation (160 mg/ml). The resulting crystals were dissolved in deionized water and ammonium sulfate added in three portions, 8 h apart, to give a solution 33% saturated (230 mg/ml). The crystals formed were redissolved and specific activity calculated as was done with each crystallization.

Histidine reactive sites

1 g of 5-aminotetrazole monohydrate (purchased from Aldrich Chemical Co.) was dissolved in 23 ml of 1.6 M HCl. To this, 0.7 g of $NaNO_2$ in 10 ml water was added slowly while in a cold room (4°). The pH was adjusted to approx. 5 with concentrated KOH solution. This diazotization procedure is from HORINISHI *et al.*[13].

A sample of diazo-tetrazole solution was mixed with an excess of histidine in 0.05 M glycine–NaOH buffer (pH 8.8). This solution, after standing at room temperature for 30 min, showed strong absorption at 360 mμ indicating that the diazo-tetrazole did react with the histidine.

After HORINISHI *et al.*[13], 1 ml of the prepared diazo-tetrazole solution was mixed with 9 ml of enzyme solution in 0.05 M buffer (pH 8.8). A 9-ml sample of the same enzyme solution was mixed with 1 ml of deionized water as a control. Both control and diazo-tetrazole–enzyme solutions were allowed to stand at room temperature for 40 min to allow for a complete reaction. The two solutions of 10 ml each were added to respective 2-g quantities of Lyphogel and allowed to stand in a cold room for 3 h and 20 min. Each solution was filtered through Whatman No. 42 filter paper into 10-ml graduated cylinders and the gel washed with distilled water.

RESULTS AND DISCUSSION

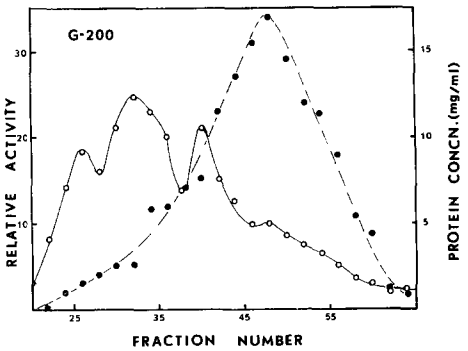
Extraction and purification

A list of typical values obtained during the extraction and purification is shown in Table I. As indicated a purification of over 26 times from the first ammonium

TABLE I

ENZYMATIC ASSAY DURING PURIFICATION PROCEDURE

Step	*Specific activity*	*Total protein (mg)*	*Enzyme units*	$A_{280m\mu}/A_{260m\mu}$ *ratio*
1st $(NH_4)_2SO_4$ precipitate	$0.27 \cdot 10^{-2}$	14 000	37.8	0.92
Acetic acid (pH 5.5)	$0.49 \cdot 10^{-2}$	6 800	33.3	0.95
2nd $(NH_4)_2SO_4$ precipitate	$1.10 \cdot 10^{-2}$	2 900	31.9	0.99
1st Sephadex treatment (G-200)	$3.3 \cdot 10^{-2}$	940	31.0	1.28
2nd Sephadex treatment (G-150 Superfine	$7.1 \cdot 10^{-2}$	400	28.4	1.34
1st crystallization	0.269	1.34	0.36	1.39
2nd crystallization	0.179	0.93	0.17	1.47
followed by Lyphogel treatment and then 3rd crystallization	0.271	0.51	0.14	1.47



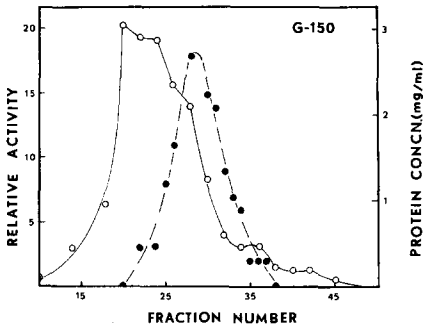


Fig. 1. Elution pattern of protein (○—○) and enzymatic activity (●—●) from Sephadex G-200.

Fig. 2. Elution pattern of protein (○—○) and enzymatic activity (●—●) from Sephadex G-150 superfine.

sulfate precipitate has been obtained. It is difficult to know the exact degree of purification from crude enzyme in liver because of the limited activity demonstrable in the acetone extract. The increase in the $A_{280\ m\mu}/A_{260\ m\mu}$ ratio indicates the loss of nucleic acid contaminant.

Typical plots of the elution patterns from Sephadex G-200 (Column I) and Sephadex G-150 superfine (Column II) are shown in Figs. 1 and 2, respectively.

Crystallization

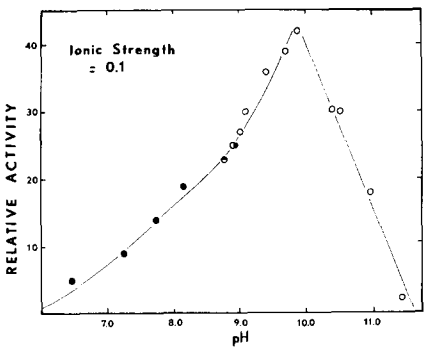
As can be seen from Table I, the first crystallization produces the greatest specific activity obtainable. There is a marked drop in total protein and enzyme units from the second Sephadex treatment due to the fact that only a limited number of fractions from the G-150 column were used in this particular example. The result of the first crystallization (0.269) is slightly higher than that obtained by METZGER *et al.*[12] (0.256), however this may reflect a species variation. It would appear that although nucleic acid has been removed in the second recrystallization some of the enzyme was denatured. Because of this, the resulting material was treated with Lyphogel and recrystallized again. The final recrystallization failed to improve the specific activity of the enzyme.

pH and ionic strength

COHEN AND ROSEMEYER[9] reported that the activity of human erythrocyte glucose-6-phosphate dehydrogenase is a function of ionic strength as well as pH. For this reason the pH dependence of enzyme activity was studied at constant ionic strength ($I = 0.1$) and constant buffer molarity (0.05 M NaOH–glycine).

Since glycine contributes little to the ionic concentration in solution when compared with the completely dissociated NaOH, all of the buffer solutions were made 0.05 M NaOH and the pH was adjusted by addition of glycine.

The constant ionic strength–pH curve (Fig. 3) shows a slow rise in activity with increasing pH to a maximum at pH 9.9 followed by a sharp drop. In the constant molarity–pH curve (Fig. 4) there is nearly the same increase in activity to the left of the maximum as in the constant ionic strength plot. However, there is a less radical drop in activity with increasing pH to the right of the optimum.



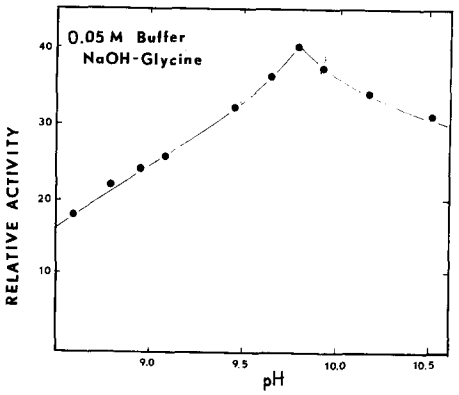


Fig. 3. Enzyme activity as a function of pH (I = 0.1) using glycine–NaOH (○—○) and Tris–malate (●—●) buffers.

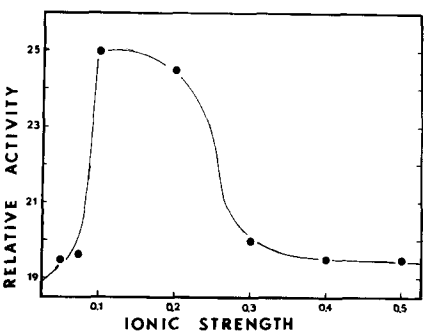
Fig. 4. Enzyme activity as a function of pH.

Although there is only a very slight change in the optimum activity, there is a significant difference in the shapes of the respective curves. This may be attributed to the fact that although the pH is becoming more unfavorable toward activity, the ionic strength is increasing as the pH increases. The combination of opposing and enhancing conditions results in a less rapid decrease in activity.

In a plot of ionic strength *versus* activity at a constant pH 9.9 (Fig. 5), there is a marked increase in activity to a maximum at I = 0.1 followed by a very slight decrease. It is therefore apparent that for optimum activity the enzyme requires the presence of ions in not less than 0.1 M ionic strength.

Temperature optimum

As with pH and ionic strength optima, there is also a temperature optimum associated with enzymatic activity. In the case of glucose dehydrogenase, we have found that this optimum temperature has a range of from 30 to 35°. The plot of temperature *versus* activity (Fig. 6) illustrates the characteristic rapid rise in activity with temperature due to the kinetic effect of temperature on reaction rate. This rise



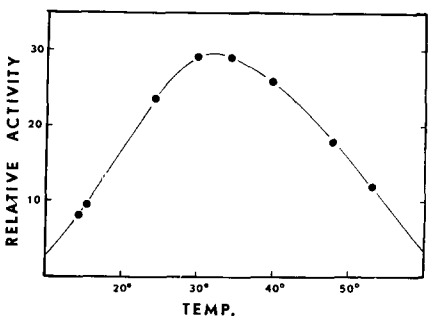


Fig. 5. Enzyme activity as a function of ionic strength in pH 9.9 glycine–NaOH buffer.

Fig. 6. Enzyme activity as a function of temperature in pH 9.9 glycine–NaOH buffer.

TABLE II

SUBSTRATE–COENZYME RELATIVE ACTIVITIES

Substrate	*NAD*	*NADP*	*NADP/NAD*
		pH = 9.1	
Glucose	22	17	0.77
Glucose 1-phosphate	0	1	—
Glucose 6-phosphate	0	2	—
		pH = 8.1	
Glucose	18	7	0.39

culminates in a broad peak followed by a slow decline in activity probably due to denaturation. It is interesting to note that the enzyme still retains some activity (approx. 7%) at 60°.

Substrate and coenzyme interrelationships

The relative activities for glucose, glucose 6-phosphate, and glucose 1-phosphate with respect to the two coenzymes, NAD and NADP, are demonstrated in Table II. As can be seen from the values for glucose, the NADP/NAD ratio is pH dependent. However, a computation of the ratio at both pH 8.1 and pH 9.1 showed a preference for NAD over NADP in both cases. This result is in contrast to that of METZGER *et al.*[12]. The pH dependence of the NADP/NAD ratio was reported earlier by STRECKER AND KORKES[3]. The table also demonstrates the very weak ability for glucose dehydrogenase to utilize glucose with phosphate in the 1 or 6 position, and it is significant to note that in both cases the only activity observed is with NADP as coenzyme.

Michaelis constants

The K_m values were determined by the method of LINEWEAVER AND BURK[11] to be 0.07 M for β-D-glucose (Fig. 7) and 0.33 μM for NAD. These values agree with those obtained by METZGER *et al.* of 0.06 M glucose for pig liver[12] and 0.38 μM NAD for rat liver[4]. Our data was collected at a pH of 9.9, $I = 0.1$ in a NaOH–glycine buffer system.

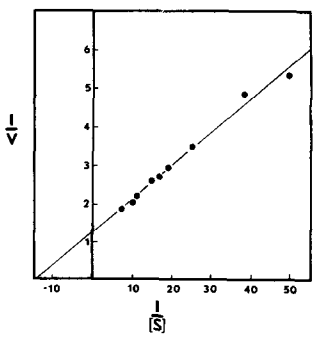


Fig. 7. Lineweaver–Burk plot.

TABLE III

COLUMN CALIBRATION

Sample	*Mol. wt.*	V_e(*ml*)
Methyl orange	327	201.0
β-Amylase	215 000	113.0
Catalase	240 000	80.8
Blue dextran (2000)	2 000 000	52.4
Glucose dehydrogenase	235 000	97.5

Molecular weight

ANDREWS[8] has shown that there is an apparent correlation between the logarithm of molecular weight of globular proteins and their elution volume (V_e) from a column of Sephadex gel. For our molecular weight determinations, solutions of blue dextran 2000 (mol. wt. 2 000 000), catalase[19] (mol. wt. 240 000), β-amylase[20] (mol. wt. 215 000), and methyl orange (mol. wt. 327) were run through a column of Sephadex G-200 as standards. The results are indicated in Table III.

The molecular weight of pig liver glucose dehydrogenase was determined to be 235 000. This is in agreement with an estimated value of 230 000 found by METZGER *et al.*[12].

Stokes radius

AKERS[15] derived an equation relating the elution volume from a Sephadex gel to the Stokes radius. He found good correlation between his data and previously determined Stokes radii by means of sedimentation studies. His relation is written

$$\frac{V_e - V_0}{V_i} = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104\frac{a}{r} + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right]$$

where V_e = elution volume, V_0 = void volume, V_i = volume of unbound solvent within the gel particles, r = effective pore radius of the gel, and a = Stokes radius. This equation was programmed for a computer and yielded a table of correlations between values for a/r and $(V_e - V_0)/V_i$. Location of the experimentally determined column parameter, $(V_e - V_0)/V_i$, for a molecule of known Stokes radius, in this case catalase with $a = 5.22$ mμ (ref. 18), gives a value for a/r. Solution for the effective pore radius followed by the similar location of a/r for an unknown macromolecule and subsequent substitution of r gives the Stokes radius, a, of the unknown molecule. The values determined on a column of Sephadex G-200 are contained in Table IV. The Stokes radius was determined to be 4.02 mμ for glucose dehydrogenase. This,

TABLE IV

STOKE'S RADIUS DATA

Sample	$\frac{V_e - V_o}{V_i}$	$\frac{a}{r}$	*a*(mμ)
Catalase	0.1910	0.312	5.22
Glucose dehydrogenase	0.3033	0.240	4.02

when compared to a value of 5.22 for catalase with a molecular weight only 2.1% greater than that determined for glucose dehydrogenase indicates that the molecule is rather compact.

Histidine reactive site

BAK AND SATO[14], in No. IV in a series of articles on *Aspergillus oryzae* glucose dehydrogenase, reported that the enzyme is sensitive to heavy metal ions such as Cu^{2+}. This is an indication that the active site on the enzyme is associated with sulfhydryl groups. However, they found that "typical sulfhydryl reagents such as *p*-chloromercuribenzoate exert no inhibitory effects". From this they conclude that activity is not related to sulfhydryl groups. Acting on the possibility that the heavy metal inactivation of the enzyme was due to complexation with the imidazole group of histidine, they utilized a procedure devised by HORINISHI *et al.*[13] for the conversion of protein histidine units to histidine bisazo-1-H-tetrazole. This process effectively blocked all of the histidine to substrate. Thus, if histidine was indeed either the active site or a part of the active site on an enzyme, then activity would be extinguished. BAK AND SATO[14] did succeed in eliminating enzymatic activity. This conclusion is supported by the earlier work of LEWIN[21] in which he found that histidine combines strongly with hexoses and pentoses.

Liver glucose dehydrogenase is known to be inhibited by heavy metal ions such as Zn^{2+} (ref. 4), and Fe^{2+} (ref. 7) and Cu^{2+} (ref. 7). It is also known to be uninhibited by the typical sulfhydryl reagent, *p*-mercurobenzoate. Although the enzyme studied by BAK AND SATO[14] is different from NAD(P)-linked glucose dehydrogenase in that it contains 1 mole of FAD as prosthetic group and is incapable of utilizing pyridine nucleotides as coenzymes, it was decided to use a modification of their procedure in an attempt to determine whether or not histidine is related to the active site.

The modified procedure was followed and it was shown that enzyme activity was consistently reduced to between 0 and 10% of its original activity. This supports the concept that histidine groups are at least in part associated with the activity of the enzyme.

ACKNOWLEDGEMENTS

The authors wish to thank the University Research Fund for financial support. In addition, Dr. M. ZANDLER is thanked for programming the necessary equations and the University Computer Center is thanked for its generous donation of computer time.

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