Beef Liver Glucose Dehydrogenase

I. Purification and Properties

NORMAN G. BRINK*

Biokemiska Avdelningen, Medicinska Nobelinstitutet, Stockholm, Sweden

Liver glucose dehydrogenase (GDH) was discovered by Harrison¹ and partially purified by him and by a number of other workers²⁻⁵. It is a diphosphopyridine nucleotide (DPN)-linked enzyme⁶, but it has been reported by several investigators that triphosphopyridine nucleotide (TPN) can replace DPN as the coenzyme^{5,7-9}. Further purification of the glucose dehydrogenase from beef liver is reported here, together with some other information concerning its properties and possible physiological role.

EXPERIMENTAL

Enzyme Assay. The GDH was assayed spectrophotometrically by measuring the initial rate of reduction of DPN by the enzyme in solution at pH 7.6 in the presence of excess quantities of glucose and the coenzyme, as described by Strecker and Korkes⁵, and employing the enzyme unit defined by them. The protein content was determined spectrophotometrically by measurement of the absorption at 340 m μ ; an assumed absorption coefficient of $E_{1\text{cm}}^{1\%} = 10$ was used.

Purification Procedure

Beef liver which had been stored in a frozen state was allowed to thaw partially overnight at 3°, cut into *ca.* 1 cm. cubes, and homogenized in a blender with about 2 volumes of acetone. The mixture was poured with stirring into 10 volumes of acetone, filtered, and the filter cake again homogenized with acetone and poured into 10 volumes of fresh acetone. After filtration, the second filter cake was broken up into 3 volumes of ether, the mixture stirred, and filtered. All of these operations were carried out in a cold-room at -18°; the acetone and ether used were previously chilled to this temperature. The filter cake was rapidly dried to constant weight at room temperature. One kg of beef liver generally yielded 250 to 300 g of acetone powder. The dry powder could be stored for several weeks at -18° with no detectable loss of activity. The acetone powder was extracted by stirring with 8 volumes of distilled water for 45 minutes at room temperature. The resulting extract was centrifuged for 45 minutes at room temperature and 2 300

* Permanent address: Organic and Biochemical Research Dept., Merck & Co., Inc., Rahway, N. J., U.S.A.

r.p.m. and the precipitate discarded. The GDH activity of the supernatant solution was generally 20–25 units/mg, with a yield of about 14 000 units per g of acetone powder. The solution could be stored for a few days at 3° if desired.

First Ammonium Sulfate Fractionation. To each 1-liter volume of the aqueous extract was added 174 g of ammonium sulfate, and the solution brought to pH 6.8–7.2 by dropwise addition of concentrated ammonium hydroxide. The solution was allowed to stand for 15 minutes, and then centrifuged for 45 minutes at 2 300 r.p.m. The precipitate was discarded. Ammonium sulfate, 137 g per liter, was added to the supernatant, and it was again adjusted to pH 6.8–7.2. After the mixture had stood for 15 minutes, it was centrifuged for 1 hour and the supernatant discarded. The precipitate was dissolved in 0.05 M pH 7.6. phosphate buffer, using about 100 ml for each liter of the original water extract. These operations were done at room temperature. The recovery of activity was 80–85 %, at a level of 60–70 u./mg. The solution was stored at 3° overnight, but preferably not longer.

Second Ammonium Sulfate Fractionation. The cold (3–5°) solution from the above step was diluted with water to a final protein concentration of about 80 mg/ml, and treated with 61.5 g of ammonium sulfate/liter. After 15 minutes, it was centrifuged for 1 hour at 5° and 6 000 r.p.m., and the precipitate discarded. The same quantity of ammonium sulfate used before was added to the supernatant, and after standing at 5° for a few minutes the mixture was again centrifuged. The supernatant was discarded, and the precipitate dissolved in 0.05 M pH 7.6 buffer to give a final volume of not more than 40 % of that at the beginning of this fractionation step. The solution was dialyzed overnight at 3° against about 8 volumes of 0.02 M pH 7.6 buffer containing 50 mg/l of cysteine hydrochloride. This step generally gave GDH concentrates of ca. 100–120 u./mg in yields of about 80–90 %.

Third Ammonium Sulfate Fractionation. The dialyzed solution from the last step was diluted with 0.02 M pH 7.6 buffer to a protein concentration of not over 50 mg/ml. Ammonium sulfate was added to 30 % saturation, and the resulting precipitate removed by centrifugation and discarded. Amounts of saturated ammonium sulfate solution, pH 7, were added dropwise to the supernatant to give first 36 % and then 43 % saturation, and the two resulting precipitates collected by centrifugation and dissolved in 0.05 M pH 7.6 buffer, all of the operations being done at 3–5°. The two fractions were dialyzed as indicated above, with two changes of the dialysis solution. Most of the activity was usually found in the second fraction, precipitated between 36 % and 43 % saturated ammonium sulfate. This fraction ordinarily contained 40–50 % of the activity, at a level of about 350 u./mg.

Ethanol Fractionation. The dialyzed solution from the above step was made 0.1 M with respect to sodium chloride and the pH brought to 5.9–6.1 with 10 % acetic acid. It was then chilled to 0°, and fractionated with 50 % (v/v) ethanol which had previously been cooled to –18°. The ethanol solution was added slowly, so that the temperature of the mixture did not rise above –2° for the first two fractions, or above –3° to –4° for subsequent fractions. Precipitates were collected by centrifugation, dissolved in 0.05 M pH 7.6 buffer, and dialyzed as usual. In a typical experiment, a first fraction, precipitated by 0.20 volume of 50 % ethanol, was discarded. Subsequent fractions were obtained by additions of 0.10, 0.10, 0.15, and 0.20 volumes of the 50 % ethanol. Fractions 3 and 4 (usually) contained about 40 % of the activity, at about 550–600 u./mg.

The purification operations up to this point were sufficiently reproducible that they could be carried out by a technician following a standardized procedure. The subsequent steps were carefully controlled, with each fraction being assayed.

Fractionation with 4.5 M Phosphate. Material from the ethanol fractionation was further purified by fractional precipitation with 4.5 M phosphate buffer, pH ca. 7. The concentrated buffer was added dropwise to the enzyme solution until the first appearance of a precipitate, generally at a phosphate concentration of about 1.0–1.2 M. This was removed by centrifugation, and the addition of phosphate continued in increments of not more than 0.1 M; the resulting precipitates were collected and dissolved in 0.05 M buffer for assay and dialysis. In a typical fractionation, the total recovery of GDH activity was about 90 %, with about 60 % of the activity precipitated at phosphate concentrations between 1.15 and 1.30 M and at a specific activity level of ca. 850 u./mg.

Final Purification. GDH concentrates of specific activity 800–900 u./mg were next fractionated with saturated ammonium sulfate solution, this procedure generally affording

a product of 1 300–1 400 u./mg activity in some 40 % yield, as well as other fractions of lower activity. The best material was dialyzed overnight and then fractionated again with 4.5 *M* phosphate. This yielded concentrates of activity 1 900–2 200 u./mg with a recovery of about 40 %. Immediate refractionation of such products with 4.5 *M* phosphate gave GDH of specific activity 3 200–3 400 u./mg in 40 % yield, as well as some less highly active fractions.

Yields. The over-all yield from the original aqueous extract (20–25 u./mg) of the acetone powder through eight steps to a product of activity 3 400 u./mg, a roughly 150-fold purification, was about 0.5 %, when only the best fractions from each step were used for the subsequent step. However, the yield could be increased several times by reworking fractions of intermediate activity.

Stability. Solutions of the enzyme in dilute pH 7.6 buffer could be stored in the refrigerator for one or two weeks without significant loss of activity, and could be kept many weeks in the cold as a precipitate under 55 %-saturated ammonium sulfate solution. However, solutions containing small amounts of ammonium sulfate frequently lost considerable activity on standing overnight.

Michaelis Constants

Coenzymes. The determinations of the Michaelis constants for the coenzymes (DPN, TPN, deaminated DPN, see below) were carried out by measuring the initial rates of reduction of the coenzyme as indicated by the increase in absorption at 340 *mμ*, using a Beckman Model B spectrophotometer. Solutions were prepared so that they contained 0.37 *M* glucose, 0.05 *M* phosphate, pH 7.00, and varied concentrations of the coenzymes. For each coenzyme concentration, three or four 3.00 ml aliquot portions were pipetted into 1 cm cuvettes. The reaction was initiated by adding at the Beckman 200 units of GDH in a volume of 0.02 ml; the values of Δd_{340} for 15 and 30 seconds so obtained in the replicate determinations were averaged. The enzyme used generally had a specific activity of 2 000 u./mg or greater; in a few cases when 1 000 u./mg material was used, no differences could be noted. All determinations were made at 21–22°. Higher temperatures increased the initial reaction rates considerably, but had less effect on the values of the Michaelis constants so obtained.

Glucose. In this case, the solutions were 2.1×10^{-4} *M* with respect to DPN, and again 0.05 *M* in phosphate. The glucose concentration was varied between 0.03 *M* and 0.12 *M*. Otherwise, the determinations were done as described above, except that the increase in absorption of the solution at 340 *mμ* was followed for one minute.

Equilibrium Constant

Solutions were prepared to contain glucose, 0.13 *M* phosphate buffer, and 11 000 units of GDH (sp. activity 2 500 u./mg) in 2.98 ml volume in a cuvette, and their absorption spectrum determined in the range 320–360 *mμ*. The reaction was initiated by the addition of 0.02 ml of DPN solution containing sufficient DPN to give a concentration of about 4×10^{-5} *M* in cuvette; the absorption readings were corrected for the addition of such a quantity of oxidized DPN. When the absorption at 340 *mμ* had become constant (ca. 45 minutes), 0.50 ml of 20 % aqueous glucose solution was added, and the absorption again determined, with appropriate corrections for dilution and the absorption of the added glucose. Finally, the pH at equilibrium was checked. The DPN concentration of the stock solution was assayed with liver alcohol dehydrogenase according to Bonnichsen.¹⁰

RESULTS AND DISCUSSION

Purity. Strecker and Korkes⁵, using aqueous extraction of acetone powder, ammonium sulfate and pH fractionations, and calcium phosphate gel treatments, obtained GDH concentrates with a specific activity of 900–1 100 u./mg. The purification procedure described in the present paper has given material

of some three times this potency, but there is no assurance that it is homogeneous. A sample of partially purified GDH of activity 1 400 u./mg was examined by electrophoresis in 0.046 *M* phosphate buffer at pH 6.96. This revealed two major components of nearly equal size and difficultly separable from each other; and a third, quite small component, which moved much faster. It was possible to isolate the small, mobile component. This proved to contain a negligible quantity of GDH, the mixture of the two large components accounting for nearly all of the enzymic activity. On this basis, it is possible that the material further fractionated to a level of 3 400 u./mg potency was nearly pure. Attempts to crystallize the small amounts of such preparations which were obtained were unsuccessful.

It is of some interest to calculate an approximate turnover number for liver glucose dehydrogenase. By assuming an activity of 3 400 u./mg and a molecular weight of 100 000, one finds that at pH 7.6, a mole of GDH reduces about 55 moles of DPN per minute. Although the absolute value of such an estimated turnover number is open to question, it is at least evident that the enzyme, if it is nearly pure, is indeed a sluggish one. In this respect it may be compared to horse liver alcohol dehydrogenase, for which a maximal turnover number of 140 for the oxidation of ethanol has been reported¹¹. The turnover numbers for most other dehydrogenase systems are several orders of magnitude higher.

Substrate Specificity. The best preparations of Strecker and Korkes⁵ oxidized xylose at about one-fourth the rate of the oxidation of glucose, while galactose and arabinose were oxidized at about 3 to 4 percent of the glucose rate. Our preparations, tested under the usual assay conditions, oxidized xylose at about 30 percent of the rate with glucose, at several levels of purification of the enzyme. A crude (71 u./mg) GDH preparation showed an activity of 10.5 u./mg with D-arabinose; but the ability to oxidize arabinose decreased with increasing purification of the enzyme until, at levels of 2 000 u./mg and above, no oxidation of arabinose could be detected. Even the most potent preparations still retained some ability to oxidize galactose, but the results varied with different galactose samples, and may possibly be ascribable to contamination of the carbohydrate.

Coenzyme Specificity. It has been recently reported that GDH preparations of specific activity 1 000 u./mg oxidized glucose in the presence of either DPN or TPN, the relative rates being dependent on pH⁵. With the usual assay conditions of pH 7.6 and both glucose and coenzyme in excess, we could detect no difference in the rate of glucose oxidation with DPN or TPN, using a GDH preparation of 2 100 u./mg. A sample of deaminated DPN was prepared according to Kaplan, Colowick and Ciotti¹². This material was not significantly less active than DPN or TPN under the assay conditions.

The Michaelis constants for DPN, TPN, and deaminated DPN were determined at pH 7.00 and 21.5° as described in the experimental section. The results are shown in Fig. 1. It may be noted that the Michaelis constants are 4.3×10^{-6} *M* for DPN, 6.2×10^{-6} *M* for TPN, and 9.0×10^{-6} *M* for deaminated DPN. The relative activities of DPN and deaminated DPN in a number of other enzyme systems have been studied by Pullman, Colowick, and Kaplan¹³.

Fig. 1. Michaelis constants for the three coenzymes. Glucose, 0.37 M, 0.05 M phosphate buffer, pH 7.00. GDH, 67 u./ml. Temperature, 21.5°. Curve I: DPN; curve II, TPN; curve III, deaminated DPN.

pH and Initial Reaction Rates. Fig. 2 summarizes the effect of the pH on the initial reaction rates in the presence of excess DPN and two concentrations of glucose. It may be observed that with both glucose concentrations, maximum rates were observed near pH 8. The data from Fig. 2, plus additional measurements, were used to calculate Michaelis constants for glucose at the different pH's. As is shown in Fig. 3, the Michaelis constant for glucose has a minimum near pH 7, where its value is $3.1 \times 10^{-2} M$, and increases markedly below pH 6.7 and above pH 7.8. Strecker and Korkes observed values of 0.15 M at pH 7.0 and of 0.07 M at pH 7.6 and 8.2.

Fig. 2. Initial reaction velocity at different pH values. DPN, $2.1 \times 10^{-4} M$. Phosphate or pyrophosphate buffer, 0.05 M. GDH, 67 u./ml. Temperature, 21.5°.

Fig. 3. Michaelis constants for glucose at different pH values.

Equilibrium Constant. The equilibrium constant for the reaction

$$\text{Glucose} + \text{DPN} \xrightleftharpoons{\text{GDH}} \text{Gluconic acid lactone} + \text{DPNH} + \text{H}^+$$

was determined at pH 7.0 and 21°, using 3 670 units of purified GDH per milliliter. In one experiment, the initial glucose concentration was $1.33 \times 10^{-4} M$ and that of DPN, $4.04 \times 10^{-5} M$; at equilibrium, 88.5 percent of the DPN was reduced. In a second experiment, starting with $6.67 \times 10^{-5} M$ glucose and $3.94 \times 10^{-5} M$ DPN, 78.2 percent of the DPN was reduced at equilibrium. Lower glucose concentrations were not used, because of the greatly lengthened time necessary to achieve equilibrium. From these experiments, values for the equilibrium constant of 2.9 and 3.3×10^{-7} were calculated. Strecker and Korkes⁵ observed an average value of 15 for the equilibrium constant at pH 6.7, using an expression which did not include hydrogen ion concentration. Their value may be compared with those reported here by multiplying by 2×10^{-7} . This gives a value of 30×10^{-7} , approximately ten times as great as what we have observed.

The detection of an apoenzyme-reduced DPN complex by observation of a shift in the absorption maximum of reduced DPN from 340 $m\mu$ to 325 $m\mu$ in the presence of large (approximately equimolecular) amounts of enzyme was observed by Theorell and Bonnichsen^{11,14} in the case of liver alcohol

Fig. 4. Competitive inhibition of GDH system by glucose-6-phosphate. DPN, $2.1 \times 10^{-4} M$, 0.05 M phosphate buffer, pH 7.00. GDH, 67 u./ml. Temperature, 22°.

dehydrogenase; and later by Chance and Neilands¹⁵ with lactic dehydrogenase, using especially sensitive spectrophotometric apparatus. Neilands¹⁶, studying the same system with conventional equipment, failed to detect the existence of such a complex. In the equilibrium experiments described above, the molar concentration of enzyme could be estimated to have been perhaps of the order of one-tenth that of the reduced DPN. No shift in the absorption maximum of the reduced DPN could be detected.

Inhibition by Hexose Phosphates. Liver glucose dehydrogenase, according to Strecker and Korkes⁵, failed to oxidize hexose diphosphate, glucose-6-phosphate, fructose-6-phosphate and ribose-5-phosphate, and these esters inhibited the oxidation of glucose by the enzyme. No quantitative data were given. We have examined the inhibition of the GDH system by fructose-1,6-diphosphate and by glucose-6-phosphate in experiments in which DPN was present in excess and the glucose concentration was varied. As can be seen from Fig. 4, glucose-6-phosphate strongly inhibited the reaction in competition with glucose; the common intercept for the lines representing the inhibited and non-inhibited reactions demonstrates the competitive nature of the inhibition¹⁷. A similar result was obtained with fructose-1,6-diphosphate. From the values of the inhibition constants, $6.2 \times 10^{-5} M$ for the fructose ester and *ca.* $2.5 \times 10^{-6} M$ for glucose-6-phosphate, it can be seen that although the enzyme is unable to oxidize these two esters, they are bound to the same reactive centers on the enzyme surface as is glucose. Moreover, the binding is some 500 times as tight in the case of fructose diphosphate, and over 10 000 times as tight with glucose-6-phosphate, as compared with the binding to the enzyme of glucose itself.

It is of interest that in the presence of excess glucose, $10^{-3} M$ glucose-6-phosphate strongly inhibited the reaction at three concentrations of DPN but, as expected, the inhibition was noncompetitive in nature. The initial reaction rates were essentially the same for each DPN concentration employed.

The Physiological Function of GDH. It is evident from consideration of the magnitude of the estimated turnover number of the enzyme and of its large Michaelis constants for glucose, that GDH oxidizes glucose only slowly at high concentrations, and below glucose concentrations of about 10^{-2} *M*, hardly at all. Furthermore, the reaction is almost completely blocked for concentrations of glucose below about 0.05 *M* by glucose-6-phosphate at levels of approximately 10^{-5} *M*. Normal liver contains about 6 per cent of glycogen, and tissues containing glycogen always have a high equilibrium concentration of glucose-6-phosphate¹⁸. Hence it seems quite likely that the GDH in liver is incapable of oxidizing glucose to any large extent, particularly as compared with the normal pathways for glucose metabolism. We have observed that aqueous extracts of freshly ground liver were devoid of GDH activity in the usual assay, although some activity appeared after precipitation with 50 %-saturated ammonium sulfate; this may perhaps be explained in terms of the discussion above. Stetten and Stetten¹⁹, on the basis of isotopic studies, concluded that the direct oxidation of glucose by liver GDH contributes little if any gluconic acid to the body fluids.

Rather than assume that liver GDH is merely a useless artifact, it would seem preferable to suppose that the enzyme does play some significant role in the body's metabolism, possibly as a DPN-linked dehydrogenase, but with some substance other than glucose as its normal substrate.

SUMMARY

1. Beef liver glucose dehydrogenase has been purified by fractionations with ammonium sulfate, ethanol and phosphate to a specific activity of about 3 400 u./mg. There is no direct evidence that such preparations are homogeneous.

2. The best preparations are capable of oxidizing only glucose, xylose, and perhaps galactose. DPN, TPN and deaminated DPN can all serve as coenzymes.

3. The Michaelis constants for DPN, TPN and deaminated DPN have been determined at pH 7.00; and for glucose at a number of pH values. An equilibrium constant has been calculated for the reaction at pH 7.00 and a temperature of 21°.

4. Strong inhibition of the enzyme system has been observed with glucose-6-phosphate, which competes with glucose, but not with DPN, for the protein surface. Fructose-1,6-diphosphate also inhibits in competition with glucose.

5. It is suggested that the oxidation of glucose to gluconic acid lactone is not the normal metabolic role of liver glucose dehydrogenase.

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REFERENCES

1. Harrison, D. C. *Biochem. J. (London)* **25** (1931) 1016.
2. Harrison, D. C. *Biochem. J. (London)* **26** (1932) 1295.
3. Wainio, W. W. *J. Biol. Chem.* **168** (1947) 569.
4. Brunelli, E., and Wainio, W. W. *J. Biol. Chem.* **177** (1949) 75.
5. Strecker, H. J., and Korkes, S. J. *Biol. Chem.* **196** (1952) 769.
6. Andersson, B. *Hoppe-Seyler's Z. physiol. Chem.* **225** (1934) 57.
7. v. Euler, H., Adler, E., Schlenk, F., and Günther, G., *Hoppe-Seyler's Z. physiol. Chem.* **223** (1935) 120.
8. Das, N. B. *Hoppe-Seyler's Z. physiol. Chem.* **238** (1936) 269.
9. Quibell, T. H. *Hoppe-Seyler's Z. physiol. Chem.* **251** (1948) 102.
10. Bonnichsen, R. K. *Acta Chem. Scand.* **4** (1950) 714.
11. Theorell, H., and Bonnichsen, R. K. *Acta Chem. Scand.* **5** (1951) 1105.
12. Kaplan, N. O., Colowick, S. P., and Ciotti, M. M. *J. Biol. Chem.* **194** (1952) 579.
13. Pullman, M. E., Colowick, S. P., and Kaplan, N. O. *J. Biol. Chem.* **194** (1952) 593.
14. Theorell, H. 8^e *Conseil de Chimie de l'Institut International de Solvay*, Bruxelles (1950) 395.
15. Chance, B., and Neilands, J. B. *J. Biol. Chem.* **199** (1952) 383.
16. Neilands, J. B. *J. Biol. Chem.* **199** (1952) 373.
17. Massart, L. in Sumner and Myrbäck, «The Enzymes», Vol. I Part I, Academic Press, Inc., New York, 1950, p. 310.
18. Cortner, R. A. «*Outlines of Biochemistry*», Third Edition, Chapter 26, John Wiley & Sons, Inc., New York, 1949.
19. Stetten, M. R. and Stetten, D. J. *Biol. Chem.* **187** (1950) 241.

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