Purification and Properties of 6-Phosphogluconate Dehydrogenase from Sheep Liver

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(Received 9 July 1969)

A method is described for the isolation of 6-phosphogluconate dehydrogenase from sheep liver. The product appears to be homogeneous in polyacrylamide-gel electrophoresis and in sedimentation-velocity and sedimentation-equilibrium studies in the ultracentrifuge. The molecular weight is estimated as 129 000 from equilibrium sedimentation.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44) was isolated from *Saccharomyces cerevisiae* by Horecker & Smyrniotis (1951) and from *Candida utilis* by Pontremoli *et al.* (1961). The enzyme was also partially purified from *Drosophila melanogaster* by Kazazian, Young & Childs (1965). The separation of 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase activities of rat liver by ammonium sulphate fractionation, and some of the properties of the crude enzymes, were reported by Glock & McLean (1953), but the purification of 6-phosphogluconate dehydrogenase from mammalian tissue has not been described previously. The preparation of the enzyme in apparently homogeneous form from sheep liver is described in the present paper, together with studies of its molecular weight. A preliminary account of this work has been published (Villet & Dalziel, 1967).

MATERIALS AND METHODS

Coenzymes and substrates. NADP⁺, NADPH, 6-phosphogluconate (barium salt), glucose 6-phosphate and ribose 5-phosphate were obtained from Boehringer Corp. (London) Ltd., London W.5. NADP⁺ was assayed as described by Klingenberg (1963). The sodium salt of 6-phosphogluconic acid was prepared by the addition of a slight excess of Na₂SO₄ to an acidified solution of the barium salt and centrifugation. The solution was brought to pH 7.0 with 0.2 M-NaOH.

Enzyme assay. The standard assay mixture consisted of 0.5 ml. of 0.17 M-glycine adjusted to pH 8.9 with 0.2 M-NaOH, 0.2 ml. of 0.1 M-MgCl₂, 0.1 ml. of 20 mM-6-phosphogluconate and 0.1 ml. of 2 mM-NADP⁺, to which 0.1 ml. of enzyme solution was added. In preliminary studies of the enzyme content of extracts of liver tissue of several species, the assays were carried out at 30° and the initial rate of reduction of NADP⁺ was measured with an Eppendorf

spectrophotometer. All other enzyme assays were made at 25° with a Zeiss model PMQII spectrophotometer. The initial rate of reduction of NADP⁺ was obtained from the change of *E*₃₄₀ during the first 2 min. and the extinction coefficient of 6.22×10^6 cm.²/mole for NADPH (Horecker & Kornberg, 1948). A unit of enzyme activity is defined as the amount that gives a rate of reduction of 1 μmole/min. The specific activity is defined as units/mg. of protein.

Protein determination. The protein content of tissue extracts was measured by the biuret method of Gornall, Bardawill & David (1949) standardized with crystalline bovine serum albumin. Throughout the subsequent purification procedure, *E*₂₈₀ was used as a measure of protein concentration, assuming an extinction coefficient of 1 cm.²/mg. Protein analysis of the purified enzyme by the biuret method gave an extinction coefficient of 1.03 cm.²/mg.

Ion-exchangers. DEAE-Cellulose was Whatman DE-11. The exchanger was washed in turn with 0.2 M-KOH, 0.1 M-HCl, 0.2 M-KOH and 0.2 M-KH₂PO₄. The pH was then adjusted to 8.0 with 0.2 M-KOH, and finally the material was equilibrated by repeated washing with 3 mM-KH₂PO₄, adjusted to pH 8.0 with 0.2 M-KOH. CM-cellulose was prepared as described by Peterson & Sober (1956), except that the final drying steps were omitted.

Polyacrylamide-gel electrophoresis. *NN'*-Methylenebisacrylamide was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. and *NNN'*-tetramethylethylenediamine from Koch-Light Laboratories, Colnbrook, Bucks. Other chemicals were from British Drug Houses Ltd., Poole, Dorset.

The technique was essentially as described by Ornstein (1964) and Davis (1964), but spacer gels were not used. The reaction mixture for the preparation of the gel consisted of 4% (w/v) acrylamide, 0.15% *NN'*-methylenebisacrylamide and 0.03% (NH₄)₂S₂O₈ in the appropriate buffer. The latter contained, in 100 ml., 6 g. of tris and 0.8 ml. of *NNN'*-tetramethylethylenediamine, together with 30 ml. of 1 M-HCl for pH 8.3, or 57 ml. of 1 M-HCl for pH 6.9. The tray buffers were 0.03 M-KH₂PO₄ adjusted to pH 8.3 or 6.9 with 0.2 M-KOH. To each gel column (length 7 cm.) 30 μg. of protein was applied in 0.04 ml. of tray buffer and 0.05 ml. of 2 M-sucrose, and overlaid with the tray buffer. The gels were finally stained overnight in 0.5% Naphthalene

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Black in 7% (v/v) acetic acid, and destained by electrophoresis for 6 hr. at 10 mA with 7% acetic acid.

Ultracentrifugation. Sedimentation-velocity, sedimentation-equilibrium and diffusion measurements were performed at 20° in a Spinco model E analytical ultracentrifuge, with schlieren optics and rotor AN-D2.

RESULTS

Extraction of the enzyme from mammalian liver. Minced livers of sheep, pig and ox, and finely sliced rat livers, were suspended in 0.125 M-potassium dihydrogen phosphate adjusted to pH 7.0 with 0.2 M-sodium hydroxide, using 200 ml. of buffer/100 g. of tissue, and kept at 5°. Samples were withdrawn at intervals and assayed for enzyme activity and protein content after centrifugation at 36000g for 20 min. in the 40 rotor of a Spinco model L ultracentrifuge. From 3–50 hr. the specific activities of the extracts of minced sheep and pig liver remained practically constant at 0.032 μ mole/mg., and that of ox liver at 0.014 μ mole/mg. The specific activity of the extract of rat liver slices increased with time of extraction up to 18 hr., reaching 0.13 μ mole/mg. and a total activity of 1.3 units/ml., considerably greater than for sheep liver mince. Nevertheless, the latter was chosen as the more convenient source for large-scale work.

Homogenization of sheep liver tissue in a Waring Blender for 1 min. and extraction as described for minced tissue gave a similar initial specific activity, but this declined after 10 hr. Pig liver homogenates gave extracts of lower specific activity. Extracts of sheep liver mince with 0.15 M-potassium chloride containing 0.16 M-potassium hydrogen carbonate had lower total and specific enzyme activities than phosphate buffer extracts.

Purification of the enzyme. All steps were carried out at 0–5° unless otherwise stated.

(1) **Extraction.** Minced sheep liver (500 g.) was extracted with 1 l. of 0.125 M-potassium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA, for 12–24 hr., with occasional stirring and readjustment of pH with 0.2 M-potassium hydroxide. The extract was filtered through cheesecloth and centrifuged at 18000g for 40 min. in an MSE 17 centrifuge.

(2) **Ammonium sulphate fractionation.** The enzyme was precipitated between 54% and 63% saturation. The centrifuged extract (830 ml.) was diluted with an equal volume of the extraction buffer, and 519 g. of ammonium sulphate was added slowly, with repeated readjustment to pH 7.0 by the addition of 1 M-ammonia. The mixture was left overnight and then centrifuged at 18600g for 30 min. To the supernatant solution, 53 g. of ammonium sulphate was added to give 54% saturation, and after 30 min. the precipitate was removed by centrifugation as before and discarded. The supernatant was brought to 63% saturation

by the addition of 119 g. of ammonium sulphate and centrifuged after 30 min. The precipitate was dissolved in the minimum volume of 5 mM-potassium phosphate buffer, pH 6.7, and dialysed for 22 hr. against the same buffer (2 $\times$ 2 l.). The dialysed solution was centrifuged to remove denatured protein and filtered through cheesecloth to remove lipid.

(3) **Fractionation with ethanol.** To 88 ml. of enzyme solution, 30 ml. of 5 mM-potassium phosphate buffer, pH 6.7, and 175 ml. of 50 mM-magnesium chloride were added, and the pH was adjusted to 6.7 with aq. ammonia. The solution was cooled to –5° while 65 ml. of ethanol (90%, v/v) was added slowly. The precipitate was removed by immediate centrifugation at 5400g for 10 min. in an MSE 6L centrifuge and discarded. The enzyme was precipitated from the supernatant solution by the addition of 90 ml. of ethanol and separated by centrifuging as before. The precipitate was dissolved in ice-cold 50 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA, and dialysed against this buffer (2 $\times$ 2 l.) for 5 hr.

(4) **Heat-denaturation.** The enzyme solution was placed in a water bath at 48° for 5 min., chilled in ice and centrifuged at 24000g for 20 min. The supernatant solution was again heated at 48° for 8 min. and denatured protein again removed by centrifugation.

(5) **Chromatography on DEAE-cellulose.** The enzyme solution was dialysed for 21 hr. against 2 l. of 3 mM-potassium phosphate buffer, pH 8.0, containing 0.1 mM-EDTA; the buffer was changed once. DEAE-Cellulose was equilibrated against the same buffer. Equilibration of both the enzyme solution and the ion-exchanger was checked by pH and conductivity measurements. The ion-exchanger was packed by gravity to give a column (2 cm. $\times$ 30 cm.) with a flow rate of 100 ml./hr. The dialysed enzyme solution, containing 0.2–0.5 g. of protein, was run in. The column was washed with 3 mM-potassium phosphate buffer (100–200 ml.) and the enzyme eluted by applying a linear concentration gradient (3–30 mM buffer). Occasionally elution of the enzyme began during the washing procedure. An elution diagram is shown in Fig. 1.

(6) **Chromatography on CM-cellulose.** The combined enzyme-containing fractions from the preceding step (210 ml.) were dialysed against 4 l. of 3 mM-potassium phosphate buffer, pH 6.9, containing 0.1 mM-EDTA, and applied to a column of CM-cellulose (2 cm. $\times$ 17 cm.) previously equilibrated with this buffer. The flow rate was 50 ml./hr. The enzyme was washed in with 10 ml. of this buffer and a linear concentration gradient of the same buffer (3–50 mM) was then applied. The pure enzyme was eluted in about 70 ml. at a concentration of 0.2 mg./ml. An elution diagram is shown in Fig. 2. The

Fig. 1. Elution diagram for chromatography on DEAE-cellulose, step 5, at pH 8.0 and 2°. An enzyme solution containing 0.2 g. of protein was added to the column (2 cm. $\times$ 30 cm.). After washing with 3 mM-potassium phosphate buffer, pH 8.0, the enzyme was eluted with a concentration gradient of the same buffer, shown by the broken line. $\circ$, Protein concentration; $\bullet$, enzyme activity.

Fig. 2. Elution diagram for chromatography on CM-cellulose, step 6, at pH 6.9 and 2°. The column size was 2 cm. $\times$ 17 cm., and the protein load 30 mg. in 210 ml. of 3 mM-potassium phosphate buffer, pH 6.9. The enzyme was eluted with a linear concentration gradient of the same buffer, shown by the broken line. $\circ$, Protein concentration; $\bullet$, enzyme activity.

concentration was doubled by readsorption on a pad of CM-cellulose (2 cm. $\times$ 2 cm.) under the same conditions and elution with 0.1 M-potassium phosphate buffer, pH 7.0. Finally, the enzyme was precipitated by addition of ammonium sulphate to 90% saturation, and stored at 2°, under which conditions it is stable for months.

A summary of the purification procedure is given in Table 1.

Gel electrophoresis. In electrophoresis on polyacrylamide gel for 4 hr. at 4 mA and 100 v, the protein migrated towards the anode as a single band, a distance of 1 cm. at pH 8.3 and 0.8 cm. at pH 6.9. Experiments with reversed polarity also showed no contaminating protein.

Ultracentrifuge studies. Sedimentation-velocity measurements were made in a double-sector cell with protein concentrations of 3.20, 2.25 and 1.20 mg./ml. in 30 mM-potassium phosphate buffer, pH 7.0. The rotor speed was 59780 rev./min., and the duration was 55 min. with the highest and lowest protein concentrations and 118 min. with the intermediate concentration. The protein sedimented as a single symmetrical peak in each experiment. The values obtained for $S_{20,w}$ were 6.35, 6.55 and 7.31 s respectively with the protein concentrations stated, and extrapolation of these few data to infinite dilution gives an approximate value of $S_{20,w}^0$ of 8.2 s.

The diffusion coefficient was also measured in the ultracentrifuge in 30 mM-potassium phosphate buffer, pH 7.0, with a synthetic-boundary cell and a rotor speed of 7447 rev./min. The diffusion coefficient was calculated by the height-area method from photographs taken at 4 min. intervals. Extrapolation of the plot shown in Fig. 3 indicates a zero-time correction of 520 sec. In Table 2, the apparent diffusion coefficients calculated from each area/height measurement and the corrected times are shown. The mean value is 6.9×10^{-7} cm.²/sec., and there is no indication of a monotonic drift of the values with time. Corrections for sedimentation and salt concentration are negligible.

Sedimentation-equilibrium studies were performed in 1 mm. multichannel cells at a rotor speed of 14290 rev./min. with protein concentrations of

Table 1. Purification of 6-phosphogluconate dehydrogenase from sheep liver

For details, see the text.						
Step	Volume (ml.)	Protein (mg./ml.)	Activity (units/ml.)	Specific activity (units/mg.)	Yield (%)	Purification
(1) Extract	800	47.5	0.61	0.013	—	1
(2) (NH ₄) ₂ SO ₄ fractionation	89	40.6	4.20	0.103	76	8
(3) Ethanol fractionation	51	14.3	4.83	0.34	50	26
(4) Heat denaturation	46	11.4	5.15	0.45	48	35
(5) DEAE-cellulose eluate	210	0.17	0.68	4.0	29	315
(6) CM-cellulose eluate	70	0.18	1.37	7.6	20	585

Fig. 3. Measurement of the apparent diffusion coefficient of the enzyme. The square of the area/height ratio of schlieren peaks, recorded in the ultracentrifuge at 20° with a rotor speed of 7447 rev./min. and schlieren angle 80°, is plotted against time. The protein concentration was 6.5 mg./ml.

Table 2. *Apparent diffusion coefficient of 6-phosphogluconate dehydrogenase from measurements of boundary spreading in the ultracentrifuge at 20°*

The protein concentration was 6.5 mg./ml. in 0.03 M-potassium phosphate buffer, pH 7.0. The area/height ratios of schlieren peaks were measured from photographs taken at 4 min. intervals. The diffusion coefficient was calculated from the corrected time, estimated from Fig. 5, by using the formula:

$$D = (\text{area/height})^2 / 4\pi t$$

Time (sec.)	$10^3 (\text{Area/height})^2$ (cm. ²)	$10^7 D$ (cm. ² /sec.)
520	4.23	6.48
760	6.88	7.19
1000	8.88	7.06
1240	10.59	6.80
1480	12.69	6.84
1720	15.41	7.13
	Mean	6.9

3.20 and 6.50 mg./ml. in the same buffer as before. Photographs were taken after 130 min. and 110 min. respectively, and analysed both by the method of Lamm (1929) and by method II of van Holde & Baldwin (1958). Examples of the plots, from the slopes of which the molecular weight is calculated, are shown in Figs. 4 and 5. The plots are linear. The molecular weights, calculated with an assumed value of 0.750 ml./g. for the partial specific volume of the protein, are given in Table 3.

Tests for contaminating enzymes. The purified enzyme was tested for glucose 6-phosphate dehydrogenase activity by adding 20 μg. to a reaction

Fig. 4. Equilibrium sedimentation of 6-phosphogluconate dehydrogenase at 20° and 14290 rev./min. for 130 min. Conditions: initial protein concentration, 3.20 mg./ml.; 1 mm. multichannel cell, diaphragm angle 80°. The data are plotted by the method of Lamm (1929); dn/dr was measured as the height of the schlieren peak at increasing radial distances r .

Fig. 5. Equilibrium sedimentation of 6-phosphogluconate dehydrogenase at 20° and 14290 rev./min. for 110 min. Conditions: initial protein concentration, 6.50 mg./ml.; 1 mm. multichannel cell, diaphragm angle 80°. The data are plotted by method II of van Holde & Baldwin (1958); n_c , the refractive increment due to solute, was obtained by numerical integration of dn/dr .

mixture comprising 3.6 ml. of 0.17 M-glycine buffer, pH 8.9, 0.1 ml. of 20 mM-glucose 6-phosphate, 0.1 ml. of 2 mM-NADP⁺ and 0.2 ml. of 0.1 M-magnesium chloride. No NADPH formation could be detected with a recording fluorimeter (Dalziel, 1962).

The 6-phosphogluconate dehydrogenase reaction is readily reversible, and if phosphoribose isomerase were present in the purified enzyme it would catalyse the oxidation of NADPH by ribose 5-phosphate. No decrease of fluorescence was detected when 50 μg. of enzyme was added to a reaction mixture comprising 0.8 ml. of 0.12 M-triethanolamine buffer, pH 7.0, 0.05 ml. of 0.3 mM-NADPH, 3.0 ml. of 0.12 M-potassium hydrogen

Table 3. *Molecular weight of 6-phosphogluconate dehydrogenase from equilibrium sedimentation measurements at 20°*

Measurements of schlieren patterns obtained with two initial protein concentrations were plotted by the methods of Lamm (1929) and van Holde & Baldwin (1958), as illustrated in Figs. 4 and 5. The molecular weight was calculated as $(\text{slope} \times 4.6RT/0.25\omega^2)$ from the Lamm plots and $(\text{slope} \times RT/0.25\omega^2)$ from the van Holde & Baldwin plots. $\omega = 1496$ radians/sec. For other details see the text.

Concn. of protein (mg./ml.)	Plot	Slope	Mol.wt.
3.20	Lamm	0.65	129000
	van Holde & Baldwin	3.07	133000
6.50	Lamm	0.64	128000
	van Holde & Baldwin	2.85	124000
	Mean		129000

carbonate solution saturated with carbon dioxide, and 0.1 ml. of 0.2 M-ribose 5-phosphate.

DISCUSSION

The procedure described for the isolation of 6-phosphogluconate dehydrogenase from sheep liver gives an overall purification of 600-fold from the centrifuged extract and a yield of 13 mg. from 500 g. of tissue. There was no evidence of the presence of more than one protein in the product in electrophoresis on polyacrylamide gel, and in sedimentation-velocity, diffusion and sedimentation-equilibrium studies in the ultracentrifuge. The product also appears to be free from glucose 6-phosphate dehydrogenase and phosphoribose isomerase, which might interfere with studies of the kinetics and the overall equilibrium constant of the 6-phosphogluconate dehydrogenase reaction.

The isolation procedure is relatively simple. The heat-denaturation step gives little increase in specific activity, and one preparation from which it was omitted gave a final product of the same

specific activity. The two ion-exchangers are essentially complementary; the specific activity of the eluates from DEAE-cellulose was variable, but the same specific activity of 7.6 units/mg. was always attained after chromatography on CM-cellulose.

The average value for the molecular weight obtained from equilibrium-sedimentation measurements (Table 3) is 129000. From an approximate estimate of $S_{20,w}^0$ of 8.2 s, and an apparent value of $D_{20,w}$ of 6.9×10^{-7} cm.²/sec. measured in the ultracentrifuge, the molecular weight calculated from the Svedberg equation, assuming a partial specific volume of 0.750 ml./g. is 116000.

We thank Mr C. Teal for assistance in the ultracentrifuge studies. R.H.V. gratefully acknowledges financial support by the Wellcome Trust and the South African Council for Scientific and Industrial Research.

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