Preparation and some Properties of L-3-Glycerophosphate Dehydrogenase from Pig Brain Mitochondria

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1. A method is described for extraction and partial purification of mitochondrial L-3-glycerophosphate dehydrogenase from pig brain. 2. By the criteria that have so far been applied, the extraction and purification procedures do not modify the activity of the enzyme towards artificial electron acceptors. 3. The amounts of acid-liberatable flavine and iron in the preparation were measured. 4. A study was made of the effects of various analogues of L-3-glycerophosphate on the activity of the enzyme.

Mitochondrial L-3-glycerophosphate dehydrogenase (EC 1.1.99.5) has been found by a number of workers to be tightly bound to the insoluble portion of the mitochondrion and, once detached from these structures, to be rather difficult to purify. Tung, Anderson & Lardy (1952) purified the enzyme 1.6-fold after extraction of a particulate fraction of rabbit skeletal muscle with deoxycholate. A rather more stable preparation was obtained by Ling, Wu, Ting & Tung (1957), using deoxycholate and trypsin as solubilizing agents and pig brain as a source. Ringler & Singer (1958) found that the phospholipase A of *Naja naja* venom would cause the release of the enzyme in soluble form from an acetone-dried powder of pig brain mitochondria. Ringler (1961) purified this form of the enzyme tenfold compared with the specific activity of the mitochondrial acetone-dried powder, and determined the quantities of flavine and non-haem iron present in the preparation.

The reasons for undertaking the development of a new purification procedure were twofold. First, the phospholipase A extraction used by Ringler & Singer (1958) causes a modification of the enzyme activity that manifests itself as a decrease in $V_{\max}$ with the artificial electron acceptors PMS† and ferricyanide. Secondly, the method of Ringler (1961) is rather time-consuming and for that reason is unsuited for the isolation of this rather unstable enzyme.

The method of purification described here yields a product that is probably of a similar degree of

purity to that described by Ringler (1961) but of a higher specific activity, since there appears to be no inactivation during the extraction procedure. This method is based on the extraction of the enzyme with the non-ionic detergent Triton X-100. Hansford & Chappell (1967) solubilized the glycerophosphate dehydrogenase of blowfly flight-muscle mitochondria with Triton X-114, although no purification of this preparation has been reported.

In an attempt to avoid modification of the enzyme activity during extraction and purification, the process was followed by using three different assay procedures to ensure that the specific activity with different electron acceptors increased in parallel.

EXPERIMENTAL

DL-3-Glycerophosphate (disodium salt, grade X), D1-glyceraldehyde 3-phosphate (monobarium salt, diethyl acetal) and D-3-phosphoglyceric acid (barium salt) were obtained from Sigma (London) Chemical Co. (London, S.W. 6). L-3-Glycerophosphate (dicyclohexylammonium salt) was obtained from Calbiochem (Los Angeles, Calif., U.S.A.). Dihydroxyacetone phosphate (dicyclohexylammonium salt, dimethyl ketal) was purchased from Boehringer Corp. (London) Ltd. (London, W. 5). L-Glyceraldehyde 3-phosphate was prepared from the racemic mixture by the method of Venkataraman & Racker (1961). Propane-1,2-diol 1-phosphate was prepared by the method of Davis & Ross (1952). DL-Glycerol 1-nitrate was the kind gift of Professor M. Dixon of this Laboratory. DCIP was obtained from British Drug Houses Ltd. (Poole, Dorset). PMS was purchased from Sigma (London) Chemical Co. Q-10, the crystalline compound from pig heart, was a kind gift from Dr O. Isler (Roche Products, Basle, Switzerland). Hydroxyapatite was purchased from Bio-Rad Laboratories (Richmond, Calif., U.S.A.) in the dried form Bio-Gel HTP, and was washed with water before use. Bovine serum albumin (fraction V) was from Sigma (London) Chemical

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† Abbreviations: PMS, phenazine methosulphate; DCIP, 2,6-dichlorophenol-indophenol; Q-6 and Q-10, ubiquinone-6 and ubiquinone-10 respectively.

Co. Triton X-100 was from Lennig Chemicals (London, W.C. 1).

Other reagents were of A.R. or highest available grade and were obtained from British Drug Houses Ltd. or Hopkin and Williams Ltd. (Chadwell Heath, Essex).

All reagents were made up in deionized water from a Permutit Series 11 deionizer. The pH of buffer solutions was determined with a Radiometer type 22 pH-meter fitted with a type B glass electrode (Radiometer, Copenhagen, Denmark) and standardized with solutions prepared from standard buffer tablets (Burroughs Wellcome and Co., London, N.W. 1).

METHODS

Glycerophosphate dehydrogenase assays. All assays were carried out at 38°, since this temperature was required for the Q-10 reduction assay and the use of the same temperature for all assays enabled direct comparisons to be made. Spectrophotometric assays were followed in a Beckman DK 2A recording spectrophotometer fitted with a constant-temperature cell housing.

(a) DCIP assay. This was a modification of the method of Ling *et al.* (1957). The reaction mixture contained 50 mM-KH₂PO₄-NaOH buffer, pH 7.6, 25 mM-L-3-glycerophosphate as the DL mixture, and 47.5 μM-DCIP, in a final volume of 2 ml. The assay was started by the addition of enzyme, and the progress of the reaction was followed by the decrease in E_{600} . The initial reaction velocity was proportional to enzyme concentration up to an extinction change of at least 0.2 E_{600} unit/min., and the progress curve was linear with time for the first minute or more throughout this range. ϵ_{600} was taken to be 2.1×10^4 cm.²/m-mole (Armstrong, 1964). A unit of glycerophosphate dehydrogenase activity in this assay is defined as the amount of enzyme that reduces 1 μmole of DCIP/min. under these assay conditions.

(b) PMS assay. The manometric assay method of Ringler & Singer (1959) was adapted for use with an oxygen electrode. The system used consisted of an oxygen electrode (Rank Bros., Bottisham, Cambs.) coupled via a sensitivity control to a 1 mV recorder (Control Instruments Ltd., Derby). The assay medium contained 50 mM-KH₂PO₄-NaOH buffer, pH 7.6, 25 mM-L-3-glycerophosphate as the racemic mixture, 17 mM-PMS and 1 mM-KCN, in a final volume of 3 ml. The medium was equilibrated with air for 5 min. and the reaction was started by the addition of enzyme. There was a lag period of about 1 min., after which the progress curve was linear until about half the oxygen in solution was exhausted. At 38°, in equilibrium with air, the oxygen concentration in the solution was taken to be 0.222 μmole/ml. A unit of enzyme activity in this assay is defined as the amount of enzyme that gives a rate of oxygen uptake of 1 μmole/min.

(c) Q-10 assay. Szarkowska & Drabikowska (1964) showed that glycerophosphate dehydrogenase in pig brain mitochondria was capable of reducing endogenous Q-10 and also that the enzyme prepared by the method of Ringler (1961) would reduce exogenous Q-6. Their assay conditions were such, however, that it was not possible to give any quantitative description of the rate of quinone reduction. Doeg, Krueger & Ziegler (1960) and Szarkowska & Drabikowska (1964) used Triton X-100 to stabilize Q-6 or Q-10 suspensions. The method described here was based on these two methods, except that the conditions of forming the Q-10 suspension were such as to result in an optically

Fig. 1. Spectrum of Q-10 in ethanol and dispersed in Triton X-100. Q-10 was 67 μM in all cases. Curve 1: oxidized Q-10 in ethanol. Curve 2: Q-10 in ethanol, reduced with KBH₄. Curve 3: oxidized Q-10 in 0.12% (w/v) Triton X-100. Curve 4: Q-10 in 0.12% (w/v) Triton X-100, reduced with KBH₄. The light-path was 0.5 cm., and the reference cell contained the solvent in all cases.

clear emulsion that could be used successfully to record the reduction of ubiquinones directly by measuring the change of E_{275} .

The basal mixture consisted of 5.0 ml. of 0.1 M-KH₂PO₄-NaOH buffer, pH 7.6, and 1.2 ml. of 1% (w/v) Triton X-100 in water. To this was added 1.0 ml. of 0.90 mM-Q-10 in ethanol, and the mixture was incubated at 38° without shaking for approx. 30 min. After this time a faintly yellow, optically clear, emulsion was produced. This emulsion was stable for at least 4 hr. if maintained at 38°, but the stability was considerably lessened if the temperature was lowered. The spectrum of Q-10 dispersed in Triton X-100 is shown in Fig. 1. Assuming a value for ΔE_{275} (oxidized—reduced) of 12.0 cm.²/μmole for Q-10 in ethanol (Lester, Hatefi, Widmer & Crane, 1959), ΔE_{275} for Q-10 dispersed in Triton X-100 was calculated to be 11.6 cm.²/μmole.

For assay of the enzyme, the reaction mixture contained, in a total volume of 1 ml., 0.72 ml. of Q-10 emulsion and 0.2 ml. of 0.125 M-L-3-glycerophosphate as the racemic mixture. The reaction was started by the addition of enzyme, and the decrease in E_{275} was measured. Because of the strong absorption by Triton X-100 in the 280 mμ region it was necessary to use 0.5 cm.-light-path cells to avoid spurious results due to stray-light effects (Mihalyi, 1965). For the conditions described here, the initial E_{275} value for the assay mixture was about 1.8. The high concentration of detergent present in the assay (0.12%, w/v) did not appear to cause inhibition of the enzyme, since the enzymic rate obtained was independent of Triton X-100 concentration between the limits of 0.08% and 0.16% (w/v). Above and below these limits the rate was slightly lowered. The progress curve was linear for at least 1 min., and the initial rate was proportional to enzyme concentration up to an extinction change of greater than 0.15 E_{275} unit/min. A unit of enzyme activity in this assay is defined as the amount of enzyme that causes the reduction of 1 μmole of Q-10/min. under these assay conditions.

Protein determinations. Protein was determined as a routine by the micro-biuret method of Goa (1953) with bovine serum albumin as standard. The bovine serum albumin used contained 9.5% (w/w) of water. No correction was made for this. Turbid samples were clarified by the

addition of Triton X-100 to a final concentration of 1.5% (w/v) before the addition of the Benedict's reagent.

Under conditions where this method was unsuitable, e.g. in the presence of high concentrations of sucrose, which interferes with the micro-biuret determination, the Kjeldahl-ninhydrin method of Jacobs (1959) was adopted; $(\text{NH}_4)_2\text{SO}_4$ was used as a standard, and the nitrogen content of the protein sample was assumed to be 16% (Bailey, 1962).

Flavine determination. FMN and FAD were determined fluorimetrically by the method of Burch (1957). The fluorimeter used was an Eppendorf spectrophotometer fitted with a fluorescence attachment. The exciting light was from the 405 and 436 m μ emission lines of a mercury lamp, and emitted light was measured after passage through a filter that was opaque to light of wavelength below 500 m μ . The instrument was calibrated by using the standard fluorescent block no. 6790. There was a linear relationship between fluorescence intensity and FMN concentration up to an FMN concentration of at least 2 μM . Concentrations of FMN as low as 2 μM could be measured to within $\pm 10\%$.

For measurement of acid-liberatable FAD and FMN in an enzyme sample the following procedure was adopted. Samples (0.4 ml.) of the protein solution, containing about 1 mg. of protein/ml., were treated in duplicate with 0.1 ml. of 50% (w/v) trichloroacetic acid. The samples were stored in the dark for 15 min. at 0°. They were then centrifuged at 10000g for 10 min. in an MSE 13 centrifuge. Samples (0.2 ml.) of the supernatants were withdrawn from each and added to 2 ml. of 0.1 M- K_2HPO_4 solution. The fluorescence of these samples was measured both before and after addition of a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$. The remainder of the supernatants was incubated overnight at 38° to hydrolyse any FAD to FMN. The next day, the fluorescence of 0.2 ml. portions was measured as before. At the final pH of the assay (pH 6.8) the fluorescence of FAD was taken to be 15% of that of the equivalent concentration of FMN. FMN standards were subjected to the whole procedure and recovery was 99%. As a routine, internal FMN standards were added to one experimental sample to check for quenching.

Total flavine was measured after digestion of 1 mg. of enzyme protein, denatured by boiling for 1 min., with 1 mg. of Pronase in a total volume of 0.5 ml. of 0.3 M- KH_2PO_4 -NaOH buffer, pH 7.6, for 4 hr. at 30°. At the end of this time protein was precipitated by the addition of 50% (w/v) trichloroacetic acid and FAD and FMN were assayed fluorimetrically in the usual way.

Iron determination. Acid-liberatable iron was measured by using a Biochemica test combination for iron [Boehringer Corp. (London) Ltd.]. This uses 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline), which was introduced for the measurement of iron by Smith, McCurdy & Diehl (1952).

Acid-liberatable iron was measured after treatment of samples of enzyme (1 ml.; 2 mg. of protein) with 0.5 ml. of 1 N-HCl for 45 min. followed by precipitation with 0.5 ml. of 1.25 M-trichloroacetic acid. Then 1.0 ml. of the bathophenanthroline reagent was added to the supernatant. Iron not liberated by this procedure was assayed by digesting the trichloroacetic acid precipitates with 1.0 ml. of 70% (w/w) HNO_3 and 0.5 ml. of 60% (w/w) HClO_4 for 8 hr. on a Kjeldahl incinerator. The residue from this digestion was diluted to 2.0 ml. with water and 1.0 ml. of the bathophenanthroline reagent was added (Peterson, 1953).

Acetone-dried mitochondria. Mitochondria were prepared from pig brain by a modification of the method of Brody & Bain (1952). All steps were carried out at 0–4° unless otherwise stated. Pig brains (1 kg.) were obtained within 1½ hr. of the death of the animals and packed in ice. The cerebral hemispheres and cerebellum were dissected free of basal structures and homogenized for 1 min. at medium speed in a Waring Blendor with 9 l. of 0.25 M-sucrose previously adjusted to pH 7.6 with 1 M- K_2HPO_4 . The homogenate was immediately readjusted to pH 7.6 with 1 M- K_2HPO_4 and centrifuged for 20 min. at 1600 rev./min. (700g) in an MSE Major centrifuge. The supernatant was decanted and centrifuged at 18000 rev./min. in an MSE 18 continuous-flow rotor, with a flow rate of 35 ml./min. This approximates to 20000g for 20 min. in discontinuous centrifugation. The white, lightly packed, upper layer of the pellet, together with the supernatant, was discarded and the tightly packed, darker-coloured mitochondrial layer was resuspended in 0.25 M-sucrose, pH 7.6, with a nylon-glass homogenizer to give a final volume about 100 ml.

This mitochondrial suspension was added drop by drop to 3.5 l. of A.R. acetone at –15° with vigorous stirring. When all the homogenate had been added, stirring was continued for a further 5 min. The suspension was filtered with suction through a double thickness of Whatman no. 1 filter paper. The resulting moist cake was resuspended in 2.5 l. of A.R. acetone at –15°, stirred for 10 min. and filtered as before. This procedure was repeated with a further 2.5 l. of acetone, and the precipitate was sucked dry on a filter funnel. After drying in a stream of air at room temperature for 1 hr., the residue was finally dried *in vacuo* over paraffin-wax chippings and solid KOH. The yield was about 10 g. of dried powder.

Purification. All steps were carried out at 0–4° unless otherwise stated.

(a) Phosphate extraction. The first step of the purification procedure was the same as that used by Ringler (1961). A sample (1.8 g.) of the mitochondrial acetone-dried powder was homogenized in 60 ml. of 0.03 M- KH_2PO_4 -NaOH buffer, pH 7.6, in a Dounce homogenizer. The suspension was stirred for 20 min. and then centrifuged at 38000g for 15 min. in an MSE 18 centrifuge. The procedure was repeated once with 60 ml. of the same buffer, to ensure removal of all the soluble protein.

(b) Triton X-100 extraction. The pellet from the previous step was homogenized in a further 57 ml. of 0.03 M- KH_2PO_4 -NaOH buffer. Then 2.7 ml. of 6% (w/v) Triton X-100 in water was added, and the suspension was stirred for 20 min. The suspension was then centrifuged for 45 min. at 105000g in an MSE Superspeed 50 centrifuge. The pellet was discarded.

(c) Hydroxyapatite fractionation. The water-clear Triton extract was treated with 6 g. dry wt. of Bio-Gel HTP that had previously been washed once with 100 ml. of water. The extract was stirred with the hydroxyapatite for 1 hr. and centrifuged for 10 min. at 2000g in an MSE Major centrifuge, and the pellet was discarded.

(d) Concentration. The supernatant was concentrated to a volume of about 6 ml. by rotatory evaporation at 30°. Care was taken to avoid letting the residue evaporate to dryness in the evaporator.

(e) Storage. The enzyme preparation was stored frozen at –10° in 0.3 M- KH_2PO_4 -NaOH buffer, pH 7.6, under which conditions the activity was essentially constant for

Table 1. *Purification of mitochondrial glycerophosphate dehydrogenase*

The results shown are for a typical purification by the steps described in the text. Activity was assayed by the DCIP-reduction assay and is expressed as DCIP units.

Stage	Volume (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Specific activity (units/mg.)	Purification	Yield (%)
1. Acetone-dried powder	65	0.57	8.35	0.068	1	100
2. Phosphate-extracted acetone-dried powder	60	0.57	7.60	0.075	1.1	92
3. Whole Triton X-100 extract	56	0.60	6.84	0.088	1.3	91
4. Triton X-100 supernatant	54	0.38	0.70	0.54	8.0	55
5. Hydroxyapatite supernatant	56	0.16	0.14	1.15	17	24
6. Concentrated supernatant	7.3	1.05	0.93	1.15	17	21

Fig. 2. Effect of the [protein]/[Triton] ratio on extraction of glycerophosphate dehydrogenase from mitochondrial acetone-dried powder. Δ and $\blacktriangle$, Percentage extraction of enzyme activity; $\circ$ and $\bullet$, specific activity of extracted enzyme. Δ and $\circ$, Protein concentration constant at 7 mg./ml., Triton concentration varied. $\blacktriangle$ and $\bullet$, Triton concentration constant at 4 mg./ml., protein concentration varied. In all cases, acetone-dried powder that had been twice extracted with 0.03 M- K_2HPO_4 -NaOH buffer, pH 7.6, was extracted with Triton in the same buffer for 20 min. at 4° and then centrifuged for 45 min. at 105 000g. Glycerophosphate dehydrogenase was assayed by the DCIP-reduction assay. Protein was determined by the micro-biuret method.

10 days. Repeated freezing and thawing caused inactivation and was avoided as much as possible.

RESULTS

The results of a typical purification are shown in Table 1. The original mitochondria had the same specific activity as the acetone-dried powder. Although only approx. 50% of the total enzyme activity was extracted by this concentration of Triton X-100, the remainder could be extracted at higher Triton X-100 concentrations. Fig. 2 shows the dependence of percentage extraction and the specific activity of the extracted enzyme on the relative concentrations of protein and Triton. Results were obtained both from experiments where protein concentration was held constant and the

Triton concentration was varied and from experiments in which the opposite conditions were adopted. The coincidence of both sets of results indicates that the [Triton]/[protein] ratio is of importance with regard both to yield and to specific activity. The particular ratio chosen for the purification procedure was a compromise between yield and purification.

Of the activity lost during the treatment with hydroxyapatite, more than 70% can be eluted from the gel by two extractions with 10 ml. of 0.1 M-potassium hydrogen phosphate-sodium hydroxide buffer, pH 7.6, containing 0.5% (w/v) of Triton, so that the loss of enzyme activity reflects a loss of enzyme protein, rather than inactivation.

The co-purification of the activities towards

Table 2. *Co-purification of activities towards various electron acceptors*

Assays were carried out as described in the text. Also shown are the K_m values for DCIP: these were determined with 25 mM-L-3-glycerophosphate in 0.05 M-KH₂PO₄-NaOH buffer, pH 7.6. The temperature for all assays was 38°.

Stage	Specific activity			PMS activity DCIP activity	10 ⁶ × K_m for DCIP (M)
	(DCIP units/mg.)	(PMS units/mg.)	(Q-10 unit/mg.)		
1. Acetone-dried powder	0.068	0.2	—	2.94	87.0
2. Phosphate-extracted acetone-dried powder	0.075	0.23	—	3.08	—
3. Whole Triton X-100 extract	0.088	0.25	—	2.84	—
4. Triton X-100 supernatant	0.54	1.55	0.43	2.88	93.0
5. Hydroxyapatite supernatant	1.15	3.35	0.88	2.90	90.0
6. Concentrated supernatant	1.15	3.35	0.88	2.90	90.0

PMS, DCIP and Q-10 is shown in Table 2. It was not possible to obtain values for the specific activity in the Q-10 assay before the solubilization step owing to the particulate nature of the preparation. As an additional safeguard against modification, the apparent K_m of the enzyme for DCIP at a fixed concentration of L-3-glycerophosphate was measured at various stages of the purification procedure. These values are also shown in Table 2.

Homogeneity. Owing to the presence of the detergent and the low concentration of protein in solution, it has not so far been possible to apply any of the conventional criteria of purity. From the specific activity of the preparation it appears that the enzyme was at a very similar degree of purity to the preparation of Ringler (1961).

Flavine and iron content. By the fluorimetric assay, the enzyme preparation at a specific activity of 1.15 DCIP units/mg. contained 1 mole of acid-liberatable FAD/ $5.5(\pm 0.2) \times 10^6$ g. of protein (mean $\pm$ S.E.M. of four determinations on different enzyme samples). The FMN content observed was rather more variable and values were obtained ranging from 1 mole of FMN/ 3.0×10^7 g. of protein to 1 mole/ 8.3×10^7 g. of protein. It seems likely that this variation was due to varying degrees of FAD hydrolysis during the assay procedure.

The values obtained for total flavine after Pronase digestion were identical with those obtained from equivalent samples in which acid-liberatable flavine was measured.

The FAD content of this preparation was rather lower than that reported by Ringler (1961), although the degree of purification was very similar in the two preparations. The value obtained by Ringler (1961) for the phospholipase A-extracted enzyme was 1 mole of FAD/ 2.1×10^6 g. of protein. This finding, together with the rather small quantity of FAD present, led to doubts as to whether the flavine was associated with the glycerophosphate dehydrogenase or with a contaminant. To test this, a sample of the enzyme was subjected to density-gradient centrifugation. A sample (0.3 ml.) of

enzyme containing 1.8 mg. of protein/ml. at a specific activity of 1.1 DCIP units/mg. was loaded on to each of two sucrose density gradients (Figs. 3a and 3b). The tubes were centrifuged at 65 000 rev./min. ($300\,000 g_{av.}$) for 4 hr. in a Spinco model L 2 ultracentrifuge (SW 65 L rotor). At the end of the run the rotor was allowed to coast to a stop without braking. Fractions from one gradient were assayed for enzyme activity by the DCIP assay and protein by the Kjeldahl-ninhydrin method. Fractions from the other gradient were assayed for enzyme activity and FAD. There was insufficient material on one gradient for all the parameters to be measured on the same sample. In both gradients, however, the peak of enzyme activity occurred at the same place. The protein peak trailed significantly behind the enzyme peak, but the FAD peak was symmetrically disposed beneath the glycerophosphate dehydrogenase peak.

It has not so far been possible to obtain direct evidence for the involvement of FAD in the activity of the enzyme. Precipitation with acid ammonium sulphate at pH 4.0 (Warburg & Christian, 1938) caused inactivation of the enzyme, but this was not reversible by incubation with either FAD or FMN. Since glycerophosphate dehydrogenase is rather unstable at acid pH, it seems probable that at least part of the inactivation was due to denaturation.

The preparation was found to contain 1 g. atom of acid-liberatable iron/ 3.1×10^5 g. of protein (mean of three determinations). No further iron was liberated by digestion with nitric acid-perchloric acid. The value obtained is in good agreement with that found by Ringler (1961) for his purified preparation, which contained 1 g. atom of total iron/ 3.5×10^5 g. of protein.

Substrate specificity. Green (1936) found that the particulate glycerophosphate dehydrogenase from rabbit muscle would oxidize only one of the optical isomers of glycerophosphate. This was the naturally occurring form, which was shown by Fischer & Baer (1937) to be of the L configuration.

In an assay medium containing, in a final volume

Fig. 3. Density-gradient centrifugation of the glycerophosphate dehydrogenase preparation. The gradient was linear from 5% to 20% (w/v) sucrose in 0.01 M- K_2HPO_4 -NaOH buffer, pH 7.6, containing 0.2% (w/v) Triton X-100. The total volume of the gradient was 5 ml. For other details, see the text. (a) —, enzyme activity; ----, protein concentration. (b) —, enzyme activity; ----, FAD concentration.

of 2 ml., 50 mM-potassium dihydrogen phosphate-sodium hydroxide buffer, pH 7.6, 47.5 μM -DCIP and 0.01 DCIP unit of enzyme, the following compounds showed no detectable activity as substrates when added in the quantities stated: glycerol 1-nitrate (10 μmoles of DL mixture), glyceric acid (10 μmoles of DL mixture), glycerol (100 μmoles), propane-1,2-diol 1-phosphate (10 μmoles of DL mixture), D-3-phosphoglyceric acid (10 μmoles) and DL-glyceraldehyde 3-phosphate (10 μmoles).

Also, in an assay containing 1.25 mM-L-3-glycerophosphate (as racemic mixture), 47.5 μM -DCIP, 50 mM-potassium dihydrogen phosphate-sodium hydroxide buffer, pH 7.6, and 0.01 DCIP unit of enzyme, in a final volume of 2 ml., the following compounds did not inhibit the reduction of DCIP: DL-glycerol 1-nitrate (10 μmoles), DL-glyceric acid (10 μmoles), glycerol (100 μmoles), DL-propane-1,2-diol 1-phosphate (10 μmoles) and dihydroxyacetone phosphate (3.5 μmoles). All these compounds also failed to inhibit in a system in which the DCIP concentration was lowered to 16 μM and the concentration of L-3-glycerophosphate was increased to 25 mM.

Fig. 4. Effect of D-3-glycerophosphate on the oxidation of L-3-glycerophosphate. Δ , Substrate L-3-glycerophosphate; $\circ$, substrate DL-3-glycerophosphate. Measurements were carried out in 0.05 M- K_2HPO_4 -NaOH buffer, pH 7.6, at a DCIP concentration of 47.5 μM with a Beckman DK 2A spectrophotometer. The temperature was 38°.

It has been pointed out by Tubbs (1962) and by Huang & Niemann (1951) that, if a substrate is contaminated with a competitive inhibitor and the

ratio of inhibitor concentration to substrate concentration is R , then:

$$\frac{1}{v} = \frac{1}{V_{\max.}} + \frac{K_m}{V_{\max.}} \left(\frac{1}{[S]} + \frac{R}{K_i} \right)$$

Therefore, if the results are plotted by the method of Lineweaver & Burk (1934), the slope of the $1/v$ against $1/[S]$ plot is unchanged and K_m and $V_{\max.}$ are both decreased by the factor $1/(1 + RK_m/K_i)$. Fig. 4 shows results of this type obtained for the racemic mixture of glycerophosphate and also for pure L-3-glycerophosphate. The D isomer was only a very weak inhibitor: K_i was calculated to be approx. 4.0×10^{-2} M.

Of the analogues of glycerophosphate that have so far been investigated, the most potent inhibitor was glyceraldehyde 3-phosphate. Fig. 5 shows that this inhibition was competitive with respect to glycerophosphate, the K_i for the racemic mixture being 6.6×10^{-5} M, determined by the method of Dixon (1953). In view of the very weak inhibition of the enzyme by D-3-glycerophosphate, it was decided to investigate the relative inhibitions due to D- and L-glyceraldehyde 3-phosphate. The sample of L-glyceraldehyde 3-phosphate used for this experiment was contaminated by 10% of the D-isomer. It was found that L-glyceraldehyde 3-phosphate was also a competitive inhibitor with

respect to glycerophosphate, and the K_i in terms of the L-isomer was 3.5×10^{-5} M.

When two competitive inhibitors, I_1 and I_2 , with inhibitor constants K_{i1} and K_{i2} compete simultaneously with the same substrate, the K_m for that substrate is increased by the factor $(1 + [I_1]/K_{i1} + [I_2]/K_{i2})$. By plotting the results by the method of Dixon (1953), plots of $[I_1]$ against $1/v$ at two substrate concentrations $[S]_1$ and $[S]_2$ will give an intersection point described by:

$$\frac{1}{[S]_1} \left(1 + \frac{[I_1]}{K_{i1}} + \frac{[I_2]}{K_{i2}} \right) = \frac{1}{[S]_2} \left(1 + \frac{[I_1]}{K_{i1}} + \frac{[I_2]}{K_{i2}} \right)$$

If $[I_2] = [I_1]/n$ at the intersection point:

$$[I_1] = - \frac{1}{\left(\frac{1}{K_{i1}} + \frac{1/n}{K_{i2}} \right)}$$

In the present case, then for $n = 1$ (racemic mixture) K_i for DL-glyceraldehyde 3-phosphate in terms of the L isomer was 3.3×10^{-5} M whereas, for $n = 9$ (L-isomer with 10% of D-isomer) K_i in terms of the L isomer was 3.5×10^{-5} M. This gave a true value of K_i for the L isomer of 3.5×10^{-5} M, whereas K_i for the D isomer was approx. 5.0×10^{-4} M.

As was found by Green (1936) for glycerophosphate dehydrogenase from rabbit muscle, D-3-phosphoglyceric acid is also an inhibitor, albeit a weak one. The inhibition is competitive with respect to glycerophosphate and K_i is 14×10^{-3} M.

DISCUSSION

The method of partially purifying glycerophosphate dehydrogenase described here appears to avoid the modification of activity that occurs during extraction of the enzyme with phospholipase A. There is no change in the total activity of the enzyme with respect to DCIP or PMS during the solubilization step, and the apparent affinity of the enzyme for DCIP is also constant throughout the purification procedure. The rather low yield obtained by the method can be attributed to loss of enzyme protein, rather than to inactivation. However, although the product of the procedure is apparently in its native state, the method has the disadvantage that no means has yet been found for removing the detergent while maintaining the enzyme in solution.

It was stated by Ringler & Singer (1962) that the specific activity obtainable by their procedure was very much dependent on the specific activity of the starting material. The same is true, within limits, of the method described here. The specific activity of the product is unaffected if the acetone-dried powder has a specific activity of more than 0.05 DCIP unit/mg., but below this value the final

Fig. 5. Inhibition by DL-glyceraldehyde 3-phosphate. Reduction of DCIP was followed at 38° in 0.05 M- K_2HPO_4 -NaOH buffer, pH 7.6. The DCIP concentration was $50 \mu M$. Glycerophosphate was the racemic mixture. DL-Glyceraldehyde 3-phosphate concentrations were: $\square$, 0; $\circ$, 0.084 mM; $\bullet$, 0.17 mM; $\triangle$, 0.28 mM; $\blacktriangle$, 0.73 mM.

activity obtainable decreases more or less in proportion. Some improvement can be obtained in these cases by increasing the amount of hydroxyapatite used, although this causes a decrease in the overall yield.

The quantities of flavine and iron present in the preparation are rather low compared with the amounts present in succinate dehydrogenase and NADH dehydrogenase (e.g. Lusty, Machinist & Singer, 1965). Also, although the concentration of iron present is very similar to that in the phospholipase-extracted enzyme, the concentration of flavine is somewhat less. What flavine there is, however, travels with the enzyme activity on density-gradient centrifugation. This suggests that at present the preparation is still far from pure, although all attempts at further purification have failed.

No substrate has so far been found that will replace L-3-glycerophosphate as the electron donor for the enzyme. However, from the study of the inhibition characteristics of substrate analogues some of the structural requirements for substrate binding can be deduced. The first of these is that there is stereospecificity for the configuration about C-2. Both D-glyceraldehyde 3-phosphate and D-3-glycerophosphate are rather weak inhibitors, as is D-3-phosphoglyceric acid, although in the last case no comparison is possible with the L-isomer. An oxygen atom is required at C-1, since propane-1,2-diol 1-phosphate does not inhibit, whereas an alcohol, aldehyde or carboxyl group in this position allows binding to take place. A relatively specific requirement for a phosphate group is also probable, since none of the non-phosphorylated compounds tried were inhibitors of, or substrates for, the enzyme. A rather surprising finding was that the product, dihydroxyacetone phosphate, was not an inhibitor. This is in accord with the findings of Estabrook & Sacktor (1958), working on the mitochondrial glycerophosphate dehydrogenase from housefly flight muscle, but is at variance with those of Ringler & Singer (1959) for the pig brain enzyme extracted with phospholipase A, for which dihydroxyacetone phosphate was found to be a competitive inhibitor with K_i 1.8×10^{-4} M.

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REFERENCES

- Armstrong, J. McD. (1964). *Biochim. biophys. Acta*, **86**, 194.
- Bailey, J. L. (1962). *Techniques in Protein Chemistry*, p. 300. London: Elsevier Publishing Co.
- Brody, T. M. & Bain, J. A. (1952). *J. biol. Chem.* **195**, 685.
- Burch, H. B. (1957). In *Methods in Enzymology*, vol. 3, p. 960. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Davis, W. & Ross, W. C. J. (1952). *J. chem. Soc.* p. 4296.
- Dixon, M. (1953). *Biochem. J.* **55**, 170.
- Doeg, K. A., Krueger, S. & Ziegler, D. M. (1960). *Biochim. biophys. Acta*, **41**, 491.
- Estabrook, R. W. & Sacktor, B. (1958). *J. biol. Chem.* **233**, 1014.
- Fischer, H. O. L. & Baer, E. (1937). *Naturwissenschaften*, **25**, 589.
- Goa, J. (1953). *Scand. J. clin. Lab. Invest.* **5**, 218.
- Green, D. E. (1936). *Biochem. J.* **30**, 629.
- Hansford, R. G. & Chappell, J. B. (1967). *Biochem. biophys. Res. Commun.* **27**, 686.
- Huang, H. T. & Niemann, C. (1951). *J. Amer. chem. Soc.* **73**, 1541.
- Jacobs, S. (1959). *Nature, Lond.*, **183**, 262.
- Lester, R. L., Hatefi, Y., Widmer, C. & Crane, F. L. (1959). *Biochim. biophys. Acta*, **33**, 169.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Ling, K. H., Wu, S. H., Ting, S. M. & Tung, T. C. (1957). *Proc. int. Symp. Enzyme Chemistry, Tokyo and Kyoto*, p. 260. London: Pergamon Press Ltd.
- Lusty, C. J., Machinist, J. M. & Singer, T. P. (1965). *J. biol. Chem.* **240**, 1804.
- Mihalyi, E. (1965). *Arch. Biochem. Biophys.* **110**, 325.
- Peterson, R. E. (1953). *Analyt. Chem.* **25**, 1337.
- Ringler, R. L. (1961). *J. biol. Chem.* **236**, 1192.
- Ringler, R. L. & Singer, T. P. (1958). *Biochim. biophys. Acta*, **29**, 661.
- Ringler, R. L. & Singer, T. P. (1959). *J. biol. Chem.* **234**, 2211.
- Ringler, R. L. & Singer, T. P. (1962). In *Methods in Enzymology*, vol. 5, p. 432. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Smith, G. F., McCurdy, W. H. & Diehl, H. (1952). *Analyst*, **77**, 418.
- Szarkowska, L. & Drabikowska, A. K. (1964). *Life Sci.* **2**, 519.
- Tubbs, P. K. (1962). *Biochem. J.* **82**, 26.
- Tung, T. C., Anderson, L. & Lardy, H. A. (1952). *Arch. Biochem. Biophys.* **40**, 194.
- Venkataraman, R. & Racker, E. (1961). *J. biol. Chem.* **236**, 1876.
- Warburg, O. & Christian, W. (1938). *Biochem. Z.* **298**, 150.