The Purification of Enolase from Cerebral Tissue

By T. WOOD*

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), The Maudsley Hospital, London, S.E. 5

(Received 12 September 1963)

During the course of a study of methods for fractionating the enzyme proteins in a dilute potassium chloride extract of cerebral tissue, it was found that enolase (D-2-phosphoglycerate hydrolyase, EC 4.2.1.11) behaved during a number of fractionation procedures in a very similar manner to ATP-creatine phosphotransferase (EC 2.7.3.2), and when a partially purified preparation of the latter was fractionated on DEAE-Sephadex, three enzyme peaks were obtained, the last of which also contained enolase (Wood, 1962, 1963). Although enolase has been prepared pure from yeast, potatoes and skeletal muscle (Malmström, 1961), there is no report of its isolation from nervous tissue, so it was decided to adopt the methods previously developed and used for the purification of ATP-creatine phosphotransferase from ox brain (Wood, 1963) and apply them to the purification of enolase from this source.

EXPERIMENTAL

2-Phosphoglyceric acid (potassium salt). The barium salt of DL-2-phosphoglyceric acid (C. F. Boehringer und Soehne, Mannheim, W. Germany) was converted into the free acid by stirring a suspension of it with freshly regenerated Zeo-Karb 225 (H⁺) (The Permutit Co. Ltd., London) at room temperature. The resin was filtered off, drained and rinsed on a coarse sintered-glass funnel under suction. The filtrate and washings were combined and again treated with resin, filtered as before, and the filtrate and washings transferred to a volumetric flask. The solution was neutralized (pH 7.0; narrow-range indicator paper) with 20% (w/v) KOH, a drop of saturated K₂SO₄ solution added and the mixture left for 2 days at 5°. At the end of this time the solution was made up to the mark, mixed and any precipitated BaSO₄ centrifuged down. This stock solution (approx. 10 mM) was stored at -20°.

To determine the content of phosphoglyceric acid, a portion of the solution was diluted 50-fold and 0.3 ml. portions were pipetted into a Pyrex test tube. The solution was evaporated over a small flame, cooled, digested with H₂SO₄-HClO₄ and the phosphorus determined as described by Wood (1961).

Assay mixture. The solution contained MgSO₄ (1 mM), imidazole-HCl buffer, pH 7.2 (50 mM), DL-2-phosphoglyceric acid (potassium salt; 2 mM), EDTA (dipotassium

salt; 0.01 mM) and KCl (400 mM). It was stored frozen at -20°.

Assay procedure. The assay mixture (0.40 ml.), in a silica microcuvette, was placed in a Unicam SP. 500 spectrophotometer fitted with a diaphragm and lens attachment. The slit width was adjusted to 0.3 mm. and the 12 mm. × 4 mm. aperture of the diaphragm, in conjunction with the lens, was selected. To start the reaction, the enzyme (diluted when necessary with 10 mM-imidazole-HCl, pH 7.2) was added from a calibrated 10 μl. constriction micropipette. The stop-clock was started and the contents of the microcuvette were mixed with a 0.5 mm. diam. flexible rod made from polystyrene catheter tubing. The extinction at 240 mμ was read against water or a suitable blank at 0.5 or 1.0 min. intervals over a period of 10–20 min. The temperature of the cell holder was measured at the start and at the end of the run.

The initial velocity of the reaction was obtained from a plot of the extinction at 240 mμ against time. From the Tables of Wold & Ballou (1957a) a molar solution of phosphoenolpyruvate in 50 mM-imidazole, pH 7.2, in the presence of 1 mM-magnesium has extinction 1380 in a 1 cm. cell. Thus a rate of 1 extinction unit/min. would correspond to 0.725 μmole of phosphoenolpyruvate/ml./min. From the measured rate and the mean temperature during the run, the rate at 20° was calculated from a temperature-correction curve. One unit of enzyme is defined as the amount catalysing the formation of 1 μmole of phosphoenolpyruvate/min. at 20° under the above conditions.

The temperature-correction curve was constructed from the initial rate of the reaction at a number of temperatures in the range 17–25° measured in a standard cuvette in the Unicam SP. 500 spectrophotometer fitted with the constant-temperature cell housing attached to a Technetemp thermostatic control unit [Technetemp (Cambridge) Ltd., Duxford, Cambs.].

Measurement of protein. The protein in crude enzyme fractions was measured by the biuret procedure described by Layne (1957), crystalline bovine serum albumin being used as a standard. Concentrations of the pure enzyme or of protein fractions from a column were determined either by this method or by measuring the extinction at 280 mμ. Measurements on the pure enzyme gave a value 1.05 for the ratio concentration by biuret method (mg./ml.)/extinction at 280 mμ in a 1 cm. cell.

Chromatography on Sephadex G-75 and G-100. The sample, in a volume of 2 ml., was placed on a column (1 cm. × 12 cm.) of Sephadex G-100 (medium), and eluted with M-KCl-0.1 M-sodium acetate, pH 6.8, in the cold room. Fractions (2.5 ml.) were collected and the protein concentration was calculated from the extinction at 280 mμ, measured

* Present address: Dept. of Pharmacy, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

against the eluent. The enzyme activity in the fractions was measured as described above.

When Sephadex G-75 was used, the procedure was the same, except that 3 ml. of sample was placed on a column (1 cm. $\times$ 15 cm.) of Sephadex G-75 medium.

Electrophoresis on cellulose acetate and in starch gel. These procedures were exactly as described by Wood (1963).

Ultracentrifuge measurements. Samples were examined in a Spinco analytical ultracentrifuge (model E) in 10 mM-imidazole-HCl buffer, pH 7.2, at either 4° or 7°. A sample of fractions 45-47 (Fig. 1) was ultracentrifuged 2 days after preparation, and again after the fractions had been stored for 24 days at 5° and pH 7.2. This second sample did not contain material which had been recovered from the ultracentrifuge cell, nor had it undergone any other manipulation.

Purification of the enzyme

The procedure was similar in many respects to that previously described for ATP-creatine phosphotransferase (Wood, 1963), but included a heating step introduced by Holt & Wold (1961) for the purification of muscle enolase.

Extraction and acetone-treatment. One whole ox brain, which had been stored at -20° until required, was extracted with 10 mM-KCl-1 mM-EDTA (2 ml./g. of tissue). The extract was treated for 2-3 days with an equal volume of acetone, and the enzyme precipitated from the 50% (v/v) acetone supernatant by adding MgSO_4 to a concentration of 20 mM. The precipitate was dissolved in 70 mM-magnesium acetate, pH 9.0. All operations up to this point were carried out exactly as already described (Wood, 1963).

Heat-treatment. The magnesium acetate extract was adjusted to pH 9.0 with 0.1 N-NaOH, heated for 15 min. in a 150 ml. conical flask in a water bath at 30° and then placed in a bath at 55°, with rapid swirling of the solution. After 5 min. the flask was removed, cooled rapidly and stood in ice for 1 hr. At the end of this time, the heat-coagulated protein was removed by centrifuging for 30 min. at 15000g, leaving a clear colourless supernatant. After the heat-treatment, all succeeding operations were performed in the cold room.

Ammonium sulphate fractionation. The pH of the solution was adjusted to 9.0 with 0.5 N-NaOH and the volume measured. Solid $(\text{NH}_4)_2\text{SO}_4$ was added gradually, with stirring, to a concentration of 1.75 M (all concentrations calculated at 20°; Dixon, 1953) and the solution stirred for 1 hr. After centrifuging for 10 min. at 10000g the supernatant was collected and $(\text{NH}_4)_2\text{SO}_4$ added to 2.4 M. After 1 hr. the precipitate and supernatant were collected as before. Ammonium sulphate was added to the supernatant, to a concentration of 3.2 M, and, after 1 hr. stirring, the precipitate was collected by centrifuging at 10000g. Each precipitate was dissolved immediately in a small volume of 50 mM-ammonium citrate, pH 9.0.

Column chromatography

Column chromatography was carried out at 2° on a column (2 cm. $\times$ 10 cm.) of DEAE-Sephadex A-50 (medium, in the acetate form) as described by Wood (1963). The sample [20-100 mg. of the 1.75-2.4 M- $(\text{NH}_4)_2\text{SO}_4$ fraction] was dialysed for 12 hr. against 10 mM-ammonium citrate, pH 9.0, and then against the 'starting buffer', consisting of 5 mM-sodium acetate-1 mM-EDTA, pH 6.8. Before it was

placed on the column, the sample was adjusted to pH 9.0 with 0.2 N-NaOH.

After washing the column with 20 ml. of 'starting buffer', the sample was run on to the column and followed by 20-40 ml. of 'starting buffer'. Elution was begun with 0.20 M-sodium acetate-1 mM-EDTA, pH 5.5, and when 60-100 ml. had passed through the gradient was started. The gradient was formed from 100 ml. of 0.20 M-sodium acetate-1 mM-EDTA, pH 5.5, in the mixing flask and 100 ml. of 0.20 M-sodium acetate-0.40 M-KCl-1 mM-EDTA, pH 5.5, in the tap funnel (Wood, 1963). When peak I (Fig. 1) began to appear in the effluent, the gradient was discontinued by removing the funnel and allowing the solution remaining in the mixing flask to run through the column. Elution was then completed with 0.20 M-sodium acetate-0.40 M-KCl-1 mM-EDTA, pH 5.5.

Fractions (5 ml.) were collected in tubes containing sufficient M-NaHCO₃ to raise their pH to about 7. After mixing, the extinction of each fraction was measured against water at 280 m μ and the protein concentration was calculated. Enzyme activity was measured in selected tubes, over a 5 min. period, by the usual assay method. Samples were concentrated, when necessary, under decreased pressure by 'ultrafiltration dialysis', as described by Everall & Wright (1958) against 10 mM-imidazole-HCl, pH 7.2.

RESULTS

Enzyme assay. The assay mixture was based on that described by Holt & Wold (Holt & Wold, 1961; Wold & Ballou, 1957b) but, as suggested by Malmström (1961), it included a low concentration of EDTA to form complexes with traces of inhibitory metals. A pH of 7.2 was chosen, and, with a concentration of DL-2-phosphoglyceric acid of 2 mM, a maximal increase of 1.3 extinction units was obtained at 240 m μ . Provided that enzyme concentrations were not too high and that the phosphoenolpyruvate concentration was not more than 40% of the concentration at equilibrium, a straight line was obtained when $E_{240 \text{ m}\mu}$ was plotted against time. The activity was shown to be proportional to the enzyme concentration over a 20-fold range of dilution. The ambient temperatures at which the assays were performed covered the range 18-24°, and the method of applying the temperature correction appeared to be satisfactory, since the same enzyme sample assayed at different times at different temperatures showed the same activity within the limits of experimental error (approx. $\pm 4\%$).

Extraction procedure. The efficiency of the potassium chloride extraction was measured by resuspending the extracted residue in the same volume of extractant as was used originally, centrifuging, collecting the supernatant and resuspending the residue a second time in the same volume of extractant. The results are shown in Table 1: method (a), the enzyme content was calculated from the total enzyme recovered by

Table 1. *Distribution of liquid, protein and enolase activity after extraction of brain tissue with potassium chloride-EDTA*

One ox brain (244 g.) was homogenized in 488 ml. of 10 mM-KCl-1 mM-EDTA. The homogenate was centrifuged (90 min. at 14000g) and the residue resuspended in 488 ml. of extractant. After centrifuging a second time (90 min. at 14000g), the residue was resuspended in 488 ml. of extractant. Details of methods (a) and (b) for calculating the enzyme content of the tissue are given in the Results section. The activity of the first extract was 7.7 units/ml. N.D., Not determined.

	Volume (ml.)	Total activity (units)	Total protein (mg.)	Sp. activity (units/mg.)
First extract	405	3120	2710	1.15
Second extract	420	441	882	0.50
Resuspended residue	700	0	N.D.	0.00

Enzyme content of brain by method (a) = $3561/244 = 14.6$ units/g. of brain.
 Enzyme content of brain by method (b) = $(7.7 \times 488)/244 = 15.4$ units/g. of brain.

Table 2. *Purification of enolase from one ox brain*

The weight of the brain was 384 g. Experimental details are given in the text. N.D., Not determined.

	Volume (ml.)	Total enzyme (units)	Total protein (mg.)	Sp. activity (units/mg. of protein)	Yield (%)
10 mM-KCl-1 mM-EDTA extract	600	3700	4464	0.83	100
50% (v/v) Acetone supernatant	1050	2600	720	3.6	70
70 mM-Magnesium acetate extract (unheated)	47	2250	458	4.9	61
70 mM-Magnesium acetate extract (after heating)	47	2130	272	7.5	58
0.1-1.75M-(NH ₄) ₂ SO ₄ fraction	5	0	15	0.0	0
1.75-2.4M-(NH ₄) ₂ SO ₄ fraction	3	770	55	14.0	21
2.4-3.2M-(NH ₄) ₂ SO ₄ fraction	5	308	91	3.4	10
> 3.2M-(NH ₄) ₂ SO ₄ fraction	50	0	N.D.	0.0	0
Enzyme from column	—	—	—	30	—

successive extractions and assay of the residue; method (b) utilized the activity of the first extract and the volume of the extractant added as described by Wood (1963). The two values obtained by the two methods were in close agreement (Table 1). The enolase content of ox brain, calculated by method (b), was 13.2 ± 1.8 units/g. of tissue (mean of four preparations).

Purification of the enzyme. The progress of the purification is summarized in Table 2. Of the initial activity, 30% was lost during the acetone-treatment. This loss apparently occurred by adsorption on to precipitated protein and was not due to any deleterious effect of the acetone, since treatment with 40% (v/v) acetone resulted in only a 10% loss of activity in the supernatant, with almost complete recovery of activity when the whole 40% (v/v) acetone mixture was assayed. However, at this concentration of acetone very little protein was precipitated and only slight purification was achieved. In the preparation procedure therefore the addition of acetone to a concentration of 50% (v/v) was retained. In the heating step, consistently good recoveries ranging from 85 to 95% were obtained. Approximately 50% of the enzyme activity placed on the DEAE-Sephadex column was recovered in peaks I and II.

Fig. 1. Gradient elution of enolase (preparation 2) from a DEAE-Sephadex column by sodium acetate-KCl-1 mM-EDTA, pH 5.5. —, $E_{280\text{ m}\mu}$; ----, enzyme activity; ·····, eluent gradient.

The purest material eluted from the column, on two occasions, had specific activity 30 units/mg. of protein, when assayed immediately after preparation and before concentration. This material, as discussed below, was probably at least 90% pure and had $S_{20,w}$ 5.48s at a protein concentration 5.5 mg./ml.

Chromatography on DEAE-Sephadex. The enzyme began to come off the column at an eluent concentration of 0.30M. A protein peak of high specific activity (peak I, Fig. 1) was obtained, closely followed by a second peak of lower specific activity (peak II, Fig. 1). The specific activities of

the fractions forming the leading edge of peak I were constant (Table 3) and what was believed to be a sample of almost pure enolase was obtained from this region by combining and concentrating frac-

tions 45–47. This material had specific activity 28 units/mg. of protein after concentration, and migrated as a single band on cellulose acetate and in starch gel. From the sedimentation pattern by the measurement of areas it was estimated to contain about 10% of contaminating material (Fig. 2).

Sedimentation-velocity runs were carried out in the ultracentrifuge on material from peak I and from peak II (Fig. 2). The $S_{20,w}$ values were determined for the two principal components observed in each sedimentation pattern and the relative amounts of each, estimated by measurements of the areas under the peaks, are given in Table 4. The relative amounts of the major components visible on starch gel after electrophoresis, as estimated by inspection, are also listed, together with the specific activities of the fractions. From Table 4 it is clear that the material with $S_{20,w}$ 5.1–5.9s was the active enzyme comprising most of peak I, and

Table 3. *Specific activities of enolase fractions eluted from a DEAE-Sephadex column*

The specific activity values refer to fractions of preparation 2 and the tube nos. to Fig. 1. For experimental details, see text.

Tube no.	Sp. activity (units/mg. of protein)
42	29.2
43	28.7
45	30.5
47	27.0
49	26.2
50	28.8
51	22.5
54	16.2

Fig. 2. Sedimentation diagrams of fractions eluted from the DEAE-Sephadex column after concentration by ultrafiltration dialysis. (a) Fractions 45–47 (Fig. 1) 2 days after preparation; 62 min. after reaching the maximum speed of 59780 rev./min. Concentration of protein was 5.5 mg./ml. and the temperature was 7°. (b) Fractions 51–52 (Fig. 1) 2 days after preparation; 62 min. after reaching 59780 rev./min. Concentration of protein was 5.2 mg./ml. and the temperature was 6.5°. (c) Fractions 45–47 (Fig. 1) after storage for 24 days at 5°; 58 min. after reaching 59780 rev./min. Concentration of protein was 5.5 mg./ml. and the temperature was 4°.

Table 4. *Distribution of components and enzyme activity of peaks I and II from the DEAE-Sephadex column*

The approximate distribution of the components on starch-gel electrophoretograms was estimated by inspection, and the distribution in the sedimentation diagram was obtained from measurements of the areas under the peaks.

Fraction	Distribution on starch gel	Distribution from the sedimentation diagram	Sp. activity of fraction (units/mg.)
Preparation 1			
Peak I	70% band I, 30% trailing bands	5.1s (60%), 2.9s (40%)*	24.6
Peak II	30% band I, 70% trailing bands	5.9s (30%), 2.8s (70%)†	7.4
Preparation 2			
Peak I, tubes 45–47 (see Fig. 2a)	90% band I, 10% trailing bands	5.5s (90%)‡	28.0
Peak II, tubes 51–52 (see Fig. 2b)	80% band I, 20% trailing bands	5.7s (70%), 2.1s (30%)‡	22.5
Peak I, tubes 45–47 (see Fig. 2c)	85% band I, 15% trailing bands	5.5s (80%)‡	19.0

* Protein concn. 3.3 mg./ml.

† Protein concn. 3.8 mg./ml.

‡ Protein concn. 5.5 mg./ml.

Table 5. *Distribution of protein and enzyme activity among fractions from a Sephadex G-75 column*

Fractions 48–52 (Fig. 1), having sp. activity 18 units/mg. after 15 days' storage at 5°, were placed on a Sephadex G-75 column and eluted with M-KCl-0.1 M-sodium acetate, pH 6.8, and 2.5 ml. fractions were collected. Protein was determined by measuring $E_{280\text{ m}\mu}$ and enzyme activity was assayed as described in the text.

Fraction no. ...	5	6	7	8	9	10	11
Protein (mg./ml.)	0.01	0.02	0.55	0.23	0.08	0.05	0.03
Enzyme (units/ml.)	0.00	0.00	2.50	4.92	0.15	0.00	0.00
Sp. activity (units/mg. of protein)	0.00	0.00	4.55	21.40	1.90	0.00	0.00

peak II was due to the presence of this peak I protein admixed with some other material ($S_{20,w}$ 2.1–2.8 s). This latter material appeared to be enzymically inactive, the activity of peak II being attributable to the peak I protein which it contained. The nature of peak II protein, $S_{20,w}$ 2.1–2.8 s, is discussed below.

Fractionation on Sephadex G-100 and G-75.

(a) G-100: fractions 42–44 (Fig. 1) were assayed after 20 days' storage at 5° and found to have a specific activity of 20.2 units/mg. After placing the material on a column of Sephadex G-100 and eluting with buffer, the enzyme activity and the protein were found to be distributed symmetrically in the same single peak. The recovered material had specific activity 17.2 units/mg. (b) G-75: fractions 48–52 (Fig. 1), with activity (after 15 days' storage) 18 units/mg., were fractionated further on Sephadex G-75. The protein peak and the enzyme peak did not coincide (Table 5), the most active fraction being of greater specific activity than the starting material. From its order of elution, the contaminating material appeared to be of greater molecular size than the active protein.

Properties of the enzyme. The original extract of cerebral tissue of pH 6.8 was stored for several weeks at 5° and for months when frozen at –20° without appreciable loss of activity, and a diluted solution of the enzyme (0.75 unit/ml.; specific activity 4.5 units/mg.) showed no detectable loss in activity after 5 hr. at room temperature. The enzyme was stable to dialysis at 5° and pH 7.2, but rather less stable at pH 9.0. It was stable to heating at 55° for 5 min., but freezing and storage at –20° of the magnesium acetate extract, or other partially purified preparations of the enzyme, often resulted in a considerable drop in activity.

Highly purified preparations declined in activity rapidly and, at the same time, material having a lower sedimentation coefficient appeared and additional slow-moving bands were visible on starch-gel electrophoretograms. Thus the activity of fractions 42–44 (Fig. 1) decreased in a linear manner with time, from 29 to 20 units/mg. over 20 days when stored at 5° in 10 mM-imidazole-hydrochloric acid buffer, pH 7.0. Similarly, fractions 45–47 (Fig. 1) fell in specific activity from

Fig. 3. Effect of magnesium on the rate of the enolase reaction. The system contained DL-2-phosphoglyceric acid (dipotassium salt; 2 mM), imidazole-HCl, pH 7.2 (50 mM), EDTA (dipotassium salt; 0.01 mM), KCl (400 mM), a dialysed preparation of enolase, and varying amounts of magnesium sulphate. Temperature, 22°; pM, –log (molar magnesium concentration).

28.0 to 19.0 units/mg. after storage under these conditions for 22 days; the relative proportion of the slowly sedimenting components had doubled (Fig. 2 and Table 4), and a small amount of more slowly moving material was noted after electrophoresis on starch gel. From these and similar observations with other preparations it was concluded that, on storage, purified enolase from which the magnesium had been removed by dialysis undergoes changes in tertiary structure and in its state of aggregation with a concomitant loss of enzymic activity.

The effect of the magnesium concentration on the initial rate of reaction was studied at 2 mM-DL-2-phosphoglyceric acid (Fig. 3). A sample of the enzyme, after dialysis to remove the magnesium, was completely inactive in an assay mixture not containing the metal. On addition of Mg^{2+} ions, the activity was restored. As reported by Wold &

Ballou (1957*b*) for yeast enolase, as the magnesium concentration was increased from zero the enzyme was first activated and then inhibited. Maximal activation was obtained at a magnesium concentration (1 mM) equal to the concentration of D-2-phosphoglyceric acid present in the reaction mixture containing the racemic substrate. Inclusion in the assay medium of sodium thioglycolate (1 mM) or of *p*-chloromercuribenzoic acid (0.1 mM) did not affect the activity. Measurements of the reaction rate at a number of temperatures in the range 17–25° enabled a linear Arrhenius plot to be constructed, from which an apparent energy of activation 14.3 kcal. was obtained. This value was identical with that determined by Westhead & Malmström (1957) for yeast enolase, activated by magnesium, in tris-hydrochloric acid buffer, pH 7.8.

DISCUSSION

Content of the enzyme in the brain. The figure obtained for ox brain of 13.2 units/g. of tissue may be compared with the 3.9 units/g. of rat brain and the 7.2 units/g. of mouse brain reported by Fellenberg, Eppenberger, Richterich & Aebi (1962) (1 Fellenberg unit = $1 \mu\text{mole}$ of substrate/min. at 25°). If 30 units/mg. is taken as the specific activity of pure brain enolase, the above value corresponds to 0.44 mg. of enzyme/g. of ox brain. This figure may be compared with that of 4.6 mg./g. of rabbit muscle calculated from the data of Czok & Bücher (1960). Thus in ox brain there is only about one-tenth of the amount of enolase that is present in rabbit muscle. A similar ratio was found previously for the contents of ATP-creatine phosphotransferase (Wood, 1963). Similarly, the activity of enolase in the initial potassium chloride extract was 0.82 ± 0.19 unit/mg. of protein (five preparations), corresponding to 2.7 ± 0.6 % of enolase in the total extracted protein, and the corresponding value for rabbit muscle was 12.2 % (Czok & Bücher, 1960). Taking the values for enolase and ATP-creatine phosphotransferase together, these two enzymes account for 17.9 % of the protein extracted by dilute salt solution from muscle, but for only 4.7 % of the protein extracted from ox brain.

Properties of the enzyme. The most striking property of the purified enzyme was the rapidity with which it lost its activity, and new components, detectable by electrophoresis or in the ultracentrifuge, made their appearance. Because of these changes, in the absence of a method of stabilizing the enzyme, an attempt to measure the molecular weight by sedimentation equilibrium was unsuccessful, owing to the progressive aggregation of the molecules. Similar changes to those reported here have been described recently by Rosenberg

(1963) and by Deal, Rutter, Massey & Van Holde (1963). Rosenberg reported what he believed to be an unfolding of the molecule on decreasing pH from 6 to 5, and Deal *et al.* (1963) discovered that crystalline yeast enolase ($S_{20,w}$ 5.85s; mol.wt. 67500) underwent a reversible association at pH 2.6–2.9 to form an enzymically inactive, highly unfolded dimer ($S_{20,w}$ 2.30s; mol.wt. 121000–127000). The similarity between these observations and the behaviour of the brain enzyme is very striking, and it is suggested that, on standing at pH 7.0, the pure magnesium-free brain enzyme ($S_{20,w}$ 5.1–5.9s) underwent unfolding and association to form an inactive dimer or higher polymer. The change was believed to be one of association rather than dissociation, despite the lower sedimentation coefficient of the product, because, on standing for longer periods, material devoid of enzyme activity was obtained which sedimented rapidly to the bottom of the cell in the ultracentrifuge. The occurrence of such changes in the physical state of the enzyme during the final stages of purification may account for the presence of peak II in the elution pattern from DEAE-Sephadex. In this peak there appeared to be active enzyme mixed with inactive material of $S_{20,w}$ 2.1–2.9s. This inactive material was eluted from a Sephadex G-75 column before the active enzyme (Table 5) and had therefore presumably a greater molecular size and weight, as would be expected if the mode of formation outlined above is correct.

The specific activity of the brain enzyme was compared with the values for enolase preparations determined by different workers at similar concentrations of substrate and activating ion and at pH 6.8–7.4 (Table 6). The specific activity of the enzyme from ox brain fell within the range of values (22–53 units/mg.) for the enzyme from rabbit muscle. However, it seemed remarkable that there should be such a lack of agreement on the value for the enzyme from this latter source. Since slight changes in the conformation of the enzyme protein can so readily occur, the variable number of components found by Malmström (1961) in some preparations of crystalline yeast enolase and the three components seen by Czok & Bücher (1960) in their crystalline enolase from muscle may represent slightly modified forms of the native molecule. If these modifications are accompanied, as seems likely, by alterations in enzymic activity, then pure specimens of enolase from rabbit muscle or yeast, prepared by different workers using slightly different techniques, may well differ in specific activity even when assayed at the same time under the same conditions. In this connexion, it is of interest that a purified specimen of brain enolase with a specific activity of 16.4 units/mg. has been prepared in these laboratories from a mag-

Table 6. *Specific activities of pure enolase from various sources*

Specific activities are in μ moles of phosphoenolpyruvate formed/min./mg. of protein at 20°, unless otherwise stated.

Source	Sp. activity	Assay conditions	Authors
Rabbit muscle	27*	No details, 25°	Czok & Bücher (1960)
	53*	1 mM-D-2-Phosphoglyceric acid, 3 mM-Mg ²⁺ , pH 6.8, 25°	Holt & Wold (1961)
	22†	2.4 mM-DL-2-Phosphoglyceric acid, 1 mM-Mg ²⁺ , pH 7.4, 22°	Malmström (1961)
	33	2.4 mM-D-2-Phosphoglyceric acid, 2.7 mM-Mg ²⁺ , pH 7.4, 20°	Boser (1959)
Potato	2	2.4 mM-D-2-Phosphoglyceric acid, 2.7 mM-Mg ²⁺ , pH 7.4, 20°	Boser (1959)
Yeast	81†	2.4 mM-DL-2-Phosphoglyceric acid, 1 mM-Mg ²⁺ , pH 7.4, 22°	Malmström (1961)
Ox brain	30	2 mM-DL-2-Phosphoglyceric acid, 1 mM-Mg ²⁺ , pH 7.2, 20°	Wood (this paper)

* Corrected to 20° from the data of Westhead & Malmström (1957).

† Rate at 22°.

nesium acetate extract stored for 26 days at -20°. This material was eluted from a DEAE-Sephadex column as a single homogeneous peak and gave a single symmetrical peak when eluted from a column of Sephadex G-75. It sedimented as a single symmetrical peak (at a protein concentration 9.2 mg./ml.: $S_{20,w}$ 3.88 s; $D_{20,w}$ 5.03×10^{-7} cm.²sec.⁻¹; mol.wt. 69600) in the ultracentrifuge and migrated as a single band on starch-gel electrophoresis.

The fully active enzyme had $S_{20,w}$ 5.48 s (at a concentration 5.5 mg. of protein/ml.), which was close to the values 5.5-5.6 s (6-10 mg. of protein/ml.) reported by Holt & Wold (1961) for rabbit-muscle enolase, and was not greatly different from the figure 5.73 s (7 mg. of protein/ml.) reported for the enzyme from yeast (Malmström, 1957). Since the enzyme could be eluted from an anion-exchange column on lowering the pH to 5.5, it was concluded that its isoelectric point is equal to, or higher than, the value 5.5-5.8 quoted for the muscle enzyme (Holt & Wold, 1961).

Although the catalytic properties of the enzyme were not systematically investigated, it was found to be activated and inhibited by Mg²⁺ ions in a similar manner to the yeast enzyme (Wold & Ballou, 1957b), and the apparent energy of activation for the reaction was the same as for the yeast enzyme (Westhead & Malmström, 1957). It resembled the yeast and muscle enzymes, but was unlike the enolase from potatoes (Boser, 1959), in being unaffected by sulphhydryl reagents.

In conclusion, enolase may readily be prepared from ox cerebral tissue by the procedure described in this paper, and it has very similar properties to those of the enzyme from yeast or rabbit muscle. When purified, the enzyme protein readily undergoes changes in configuration and in its state of aggregation similar to those which are now being recognized as occurring with enolase from other sources.

SUMMARY

1. Enolase was extracted with potassium chloride-EDTA solution from frozen ox brains; it was present in amounts averaging 13.2 ± 1.8 units/g. of tissue and represented $2.7 \pm 0.6\%$ of the protein in the extract.

2. A 35-fold purification was achieved and essentially pure enolase of specific activity 30 units/mg. was obtained after chromatography on DEAE-Sephadex.

3. The pure enzyme showed a marked tendency to undergo changes in its structure and state of aggregation, with an accompanying loss of activity.

4. The effects on the enzyme reaction of temperature, magnesium concentration and compounds reacting with sulphhydryl groups were investigated. The physical and chemical properties were very similar to those reported for enolase prepared from yeast or muscle.

The author is greatly indebted to Dr D. B. Gammack for his co-operation and the provision of the sedimentation data, which were obtained with an ultracentrifuge provided by the Medical Research Council. The author also wishes to thank Professor H. McIlwain for helpful discussion, and Mr J. Mills, who carried out some preliminary studies. Technical assistance was provided by Miss B. J. Aylett and by Mr H. Russell. The work was supported by a Research Fund Grant from the Bethlem Royal Hospital and the Maudsley Hospital.

REFERENCES

- Boser, H. (1959). *Hoppe-Seyl. Z.* **315**, 163.
- Czok, R. & Bücher, T. (1960). *Advanc. Protein Chem.* **15**, 315.
- Deal, W. C., Rutter, W. J., Massey, V. & Van Holde, K. E. (1963). *Biochem. biophys. Res. Commun.* **10**, 49.
- Dixon, M. (1953). *Biochem. J.* **54**, 457.
- Everall, P. H. & Wright, G. H. (1958). *J. med. Lab. Tech.* **15**, 209.
- Fellenberg, R. von, Eppenberger, H., Richterich, R. & Aebi, H. (1962). *Biochem. Z.* **336**, 334.

- Holt, A. & Wold, F. (1961). *J. biol. Chem.* **236**, 3227.
 Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 450. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Malmström, B. G. (1957). *Arch. Biochem. Biophys.* **70**, 58.
 Malmström, B. G. (1961). In *The Enzymes*, 2nd ed., vol. 5, p. 471. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
 Rosenberg, A. (1963). *Fed. Proc.* **22**, 346.
 Westhead, E. W. & Malmström, B. G. (1957). *J. biol. Chem.* **228**, 655.
 Wold, F. & Ballou, C. E. (1957a). *J. biol. Chem.* **227**, 301.
 Wold, F. & Ballou, C. E. (1957b). *J. biol. Chem.* **227**, 313.
 Wood, T. (1961). *J. Chromat.* **6**, 142.
 Wood, T. (1962). *Biochem. J.* **85**, 31 p.
 Wood, T. (1963). *Biochem. J.* **87**, 453.

Biochem. J. (1964) **91**, 460

The Reduction of Pantethine by an Extract of Camel Intestine

BY I. F. DURR AND N. CORTAS

Department of Biochemistry, School of Medicine, American University of Beirut, Beirut, Lebanon

(Received 21 October 1963)

Thompson, Bird & Peterson (1954) have reported that pantethine was as active as pantotheine in promoting chick growth when administered orally or intraperitoneally. This finding suggests that pantethine may be readily reduced to pantotheine. Since several mechanisms are known for the reduction of disulphides, it was decided to determine the nature of this reductive reaction.

The results presented below indicate that the reduction of pantethine occurs by the coupling of reactions (1) and (2):

Reaction (1) proceeds non-enzymically, the mercaptide ion of GSH being the reactive species. Reaction (2) is catalysed by glutathione reductase which has been partially purified from the small intestine of the dromedary camel.

MATERIALS AND METHODS

Chemicals. Pantethine, NADPH, NADP⁺, GSH, GSSG, cystine, oxidized 6,8-dimercapto-octanoic acid (α -lipoic acid) and tris were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. DEAE-cellulose (mesh no. 40) was obtained from the Brown Co., Berlin, N.H., U.S.A. Other reagents were purchased from E. Merck, Darmstadt, Germany.

Buffers. 1 M-Potassium phosphate buffer, pH 7.5, and 1 M-tris-HCl buffer were prepared as described by Gomori (1955). 0.1 M-Ammonium formate buffer, pH 5.5, was prepared by titrating 0.1 M-formic acid with 0.1 N-ammonia to pH 5.5 by using a glass electrode.

Preparation of diethylaminoethylcellulose. DEAE-cellulose (mesh no. 40) (100 g.) was suspended in 2.5 l. of distilled water. The suspension was adjusted to pH 7.5 by the addition of 130 ml. of 1.5 M-KH₂PO₄ and 150 ml. of N-KOH. The mixture was thoroughly stirred for 1 hr., then left for the sediment of DEAE-cellulose to form overnight.

The supernatant layer was decanted, and the slurry was then filtered under suction to remove residual buffer solution. The DEAE-cellulose was washed with 6 l. of distilled water and finally suspended in 2 l. of 1 M-potassium phosphate buffer containing EDTA (1 mM). A 25 ml. portion of this suspension is equivalent to 1 g. of the dry DEAE-cellulose.

Determination of the concentrations of proteins, mercaptans and reduced nicotinamide-adenine dinucleotide phosphate. The concentration of proteins was determined spectrophotometrically (Warburg & Christian, 1941). Mercaptans were determined according to the method of Grunert & Phillips (1951), crystalline GSH being used to prepare the reference curve. The NADPH and NADH were assayed spectrophotometrically at 340 m μ assuming a molecular extinction coefficient of 6220.

Assay of enzymic activity. The reduction of disulphides was spectrophotometrically determined in the presence of NADH or NADPH by following the decrease in the extinction at 340 m μ at 30°. Each cuvette contained 2.4 μ -moles of the disulphide to be tested, 0.2–1 mg. of protein, 0.3 μ mole of NADPH or NADH and 100 μ moles of potassium phosphate buffer, pH 7.5, in a final volume of 2.9 ml. Readings were taken at 1 min. intervals. A specific activity of 1 represents the oxidation of 1 μ mole of NADPH or NADH/mg. of protein/min. under the above conditions of assay.

Preparation of the enzyme. (1) Preparation of extract. Unless otherwise indicated all operations were carried out at 7°. The small intestine (34 m.) of a freshly slaughtered camel *Camelus dromedarius* was cut into about 1 m. pieces opened lengthwise and thoroughly washed with chilled 0.1 M-KCl. The mucosa was scraped with a microscope slide and 980 g. of tissue was collected. When the mucosa was frozen at -10° the glutathione-reductase activity of the tissue was retained for at least 1 month. A portion (36 g.) of frozen mucosa was placed in a Waring Blender with 90 ml. of 0.1 M-KCl and blended at top speed for 2 min. Then another 90 ml. of 0.1 M-KCl was added and the suspension was further blended for 1 min. Centrifugation at 4000g for 20 min. yielded 193 ml. of a clear solution (9.45 mg. of protein/ml.).