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The Separation, Partial Purification and some Properties of Isoenzymes of Aldolase from Guinea-Pig Cerebral Cortex

By P. C. NICHOLAS and H. S. BACHELARD
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), De Crespigny Park, London S.E.5

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1. Aldolase isoenzymes from guinea-pig cerebral cortex were partially purified and separated by ammonium sulphate fractionation and chromatography on DEAE-cellulose. 2. Each purified isoenzyme was shown to be virtually uncontaminated with other forms by starch-gel electrophoresis. The quantitative distribution of the isoenzymes was: I, 6·2%; II, 5·2%; III, 15·3%; IV, 25·7%; V, 33·3%. 3. The pH optima for the five separated isoenzymes were similar; all were in the range pH 7·5–8·0. Values for pK_a (6·31–6·55) and pK_b (9·45–9·59) were calculated from the data and suggested the involvement of histidine and lysine residues. 4. The stabilities of the isoenzymes were shown to be I = II > III > IV > V at pH 4·4 in order of decreasing stability and are discussed in terms of subunit structure. 5. The substrate activity ratios (fructose 1,6-diphosphate/fructose 1-phosphate) were measured and all were in the range 12–44.

Liver and muscle fructose 1,6-diphosphate aldolases (EC 4.1.2.7 and EC 4.1.2.13) have been well investigated with respect to physical, chemical and catalytic properties (Peanasky & Lardy, 1958*a*,*b*; Rutter, Richards & Woodfin, 1961; Horecker, Rowley, Grazi, Cheng & Tchola, 1963; Spolter, Adelman & Weinhouse, 1965; Kawahara & Tanford, 1966).

The occurrence of multiple forms (isoenzymes) of aldolase in a variety of tissues has been noted in fowl (Herskovits, Masters, Wassarman & Kaplan, 1967) and in man, rat and frog (Anstall, Lapp & Trujillo, 1966). Five forms of aldolase have been demonstrated in rabbit brain (Penhoet, Rajkumar & Rutter, 1966; Foxwell, Cran & Baron, 1966) and ox brain (Rensing, Schmid, Christen & Leuthardt, 1967). The isoenzymes of rabbit brain have been suggested to be composed of two different subunits combined as tetramers (Penhoet *et al.* 1966; Penhoet, Kochman, Valentine & Rutter, 1967) similar to the tetramer described for the rabbit muscle enzyme (Kawahara & Tanford, 1966).

Studies on the physical and chemical properties of aldolase in the brain require a complete separation and purification of the individual types on a preparative scale. The occurrence of five distinct and identifiable forms with similar catalytic properties offers opportunities to study correlations between structure and enzymic function.

In the present work five electrophoretically distinct isoenzymes of aldolase were quantitatively separated from guinea-pig cerebral cortex. Each isoenzyme was purified to the extent that it was uncontaminated by other forms. Some properties of the isoenzymes are described and compared.

The isoenzymes are identified by numbers assigned according to the extent of anodic mobility. That with the greatest anodic mobility has been assigned the number I and the others, in order of decreasing anodic mobility, the numbers II, III, IV and V. This procedure is in accordance with the recommendations of the subcommittee on isoenzymes of the Standing Committee on Enzymes of the International Union of Biochemistry (Webb, 1964).

A preliminary account of this work has been given (Nicholas & Bachelard, 1968).

MATERIALS AND METHODS

Centrifugation. The Spinco model L2–65 ultracentrifuge was used throughout at 0–1°; *g* values are expressed as $g_{av.}$.

Preparation of tissue extracts. Guinea pigs were killed by exsanguination after being stunned by a blow on the back of the neck. The cerebral hemispheres were quickly removed, cleared of white matter and added to ice-cold homogenizing medium.

In two experiments to determine the distribution of aldolase, one hemisphere (approx. 1 g.) was added to 8·6 ml. of 0·1 M-glycine–NaOH buffer, pH 8·5, and the other to 8·6 ml. of 0·32 M-sucrose, pH 7·0. The brains were homogenized in a Teflon-pestle homogenizer (type A, clearance

0·10–0·15mm.; A. H. Thomas and Co., Philadelphia, Pa., U.S.A.) for 2min. at 1500rev./min. with intermittent periods of cooling in ice. The homogenates were centrifuged at 150000*g* for 1hr. (no. 50 rotor).

The supernatant fractions (supernatant 1) were removed, and the pellets were resuspended in 8·6ml. of the same extractant, rehomogenized by hand and centrifuged at 150000*g* for 1hr. The supernatant fractions (supernatant 2) were collected and the pellets resuspended in 8·6ml. of the corresponding extractant by homogenization by hand.

For larger-scale preparations of cerebral-cortex aldolase, pooled cerebral hemispheres were placed in ice-cold, 0·1M-glycine–NaOH buffer, pH8·25–8·50 (4ml./g. of tissue), and homogenized in a Teflon-pestle homogenizer (type C, clearance 0·15–0·23mm.; A. H. Thomas and Co.) for 2min. at 1500rev./min. with intervals in ice for cooling. The homogenate was centrifuged at 150000*g* for 1hr. and the combined supernatant fractions were used.

Ammonium sulphate fractionation. The amount of solid $(NH_4)_2SO_4$ required to alter the degree of saturation of a solution at 0° from an initial degree of saturation S_1 to a final degree of saturation S_2 was calculated from the following equation (during this study a similar equation was published by Di Jeso, 1968), similar to that described by Kunitz (1952) for $(NH_4)_2SO_4$ fractionation at 23°:

$$X = \frac{50{\cdot}5(S_2 - S_1)}{1 - 0{\cdot}285S_2}$$

where X is g. of $(NH_4)_2SO_4$/100ml. The equation is derived on the basis of the following: (i) solubility of $(NH_4)_2SO_4$ at 0° is 70·6g./100ml. of water (Hodgman, 1959); (ii) specific volume of solid $(NH_4)_2SO_4$ is 0·565ml./g. (Hodgman, 1959) (iii) volume of a solution saturated with 70·6g./100ml. of water is $100 + (70{\cdot}6 \times 0{\cdot}565)\text{ml.} = 139{\cdot}89\text{ml.}$; (iv) concentration of $(NH_4)_2SO_4$ in a saturated solution is 0·505g./ml. of solution.

The tissue supernatant fraction was brought to 0·400 saturation by the addition, with constant stirring at 0°, of $(NH_4)_2SO_4$ (22·800g./100ml.). The pH of the mixture was maintained at pH7·5–8·0 with 0·5M-NaOH. After the complete addition of $(NH_4)_2SO_4$ the mixture was allowed to equilibrate for 15min. and then centrifuged at 34880*g* for 15min. (no. 30 rotor).

The supernatant was removed from the precipitate by decantation and adjusted to 0·500 saturation by the addition of 5·8166g. of $(NH_4)_2SO_4$/100ml. After equilibrating for 15min. the mixture was centrifuged at 34880*g* for 15min. and the supernatant collected by decantation.

The degree of saturation was then increased progressively from 0·5 saturation in steps of 0·025 to 0·675 saturation by the addition of 1·4846g./100ml. (0·525), 1·4971g./100ml. (0·550), 1·5100g./100ml. (0·575), 1·5229g./100ml. (0·600), 1·5360g./100ml. (0·625), 1·5500g./100ml. (0·650) and 1·5631g./100ml. (0·675). After each complete addition of $(NH_4)_2SO_4$ the mixture was equilibrated and centrifuged and the supernatant collected as described above.

All precipitates, with the exception of that from the 0·400-saturation step, were dissolved in a total of 10ml. of 'tris buffer' (see below). The 0·400-saturation precipitate was usually dissolved in 40ml. of buffer after being homogenized at low speed (500rev./min.) to facilitate solution.

The solutions were then dialysed against six changes of 'tris buffer' at 2° over a period of 4–6hr. to remove $(NH_4)_2SO_4$ before DEAE-cellulose chromatography.

DEAE-cellulose chromatography. DEAE-cellulose (Whatman DE-52, microgranular, pre-swollen; W. and R. Balston Ltd., Maidstone, Kent) was suspended (200g./l.) in 8·4mM-NaCl and titrated to pH7·35 with 8·4mM-HCl. The DEAE-cellulose was allowed to settle and the supernatant and fines were sucked off. The material was then resuspended at least five times in 'tris buffer' and finally stored at 2°. Air and CO_2 were removed before use by application of a gentle vacuum, not sufficient to cause boiling, for 10min.

Chromatography was carried out on DEAE-cellulose columns (40cm. × 1·8cm.) at 2° that, after being poured, had been equilibrated with 'tris buffer' by pumping for 4hr. at a flow rate of about 8·5ml./hr. After dialysis, the aldolase-enriched fractions precipitated between 0·500 and 0·650 saturation with $(NH_4)_2SO_4$ were combined (total volume 50–60ml.), pipetted on to the upper surface of the DEAE-cellulose and allowed to pass into the column without pumping. Before all of the aldolase solution had passed into the column, 'tris buffer' was layered on the aldolase fraction and pumping restarted at the equilibration flow rate.

The first peak of aldolase activity was eluted by the starting buffer and elution of subsequent peaks of aldolase activity was achieved by a stepwise gradient of NaCl (0·02–0·14M) in 'tris buffer'. Fractions (10ml.) were collected with an LKB UltraRac fraction collector. Aldolase assays were carried out on all column fractions and eluting buffers were not changed until aldolase activities in the fractions had fallen to negligible values. Protein determinations were made on all fractions. The fractions were stored at 0° in 'tris buffer' or as freeze-dried powders.

Starch-gel electrophoresis. Horizontal starch-gel electrophoresis (Smithies, 1955) was performed in Perspex trays (13cm. × 21cm. × 0·8cm.) fitted with a detachable margin (0·3cm. deep) in phosphate–citrate buffer, pH7·03. After electrophoresis for 18hr. at 2–3v/cm. and 35mA the gels were sliced horizontally with fine steel wire after the detachable margin had been removed. The upper layer of gel was stained for protein in a saturated solution of Naphthalene Black 10B in methanol–water–acetic acid (5:5:1, by vol.) for 5–10min. and background colour removed with the same solvent (Smithies, 1955).

The lower layer was stained for aldolase activity by pouring a solution (50ml.) of the following composition on to the surface of the gel, after the detachable margin had been replaced, and incubation for 1–2hr. at 37°: fructose 1,6-diphosphate, pH8·25 (3mM), Na_2HASO_4 (40mM), $Na_4P_2O_7$ (40mM), NAD^+ (0·2mM), glyceraldehyde 3-phosphate dehydrogenase (0·2unit/ml.), phenazine methosulphate (0·01mg./ml.), nitro-blue tetrazolium (0·2mg./ml.) and glycine–NaOH buffer, pH8·25 (60mM). After staining, the gels were hardened by immersion in methanol–water–acetic acid for several hours and stored in the dark.

Photography. The gels were illuminated by two photoflood lamps (no. 1, 275w) and photographed on Ilford Pan-F film by using a 2X yellow filter. Development was done in Microdol-X.

pH *optima and stability experiments.* For these studies freeze-dried fractions of the enzymes were reconstituted with 'tris buffer' and dialysed twice against 500ml. volumes of the same buffer for 2hr.

In the pH–activity studies the enzyme solutions were

further diluted with 'tris buffer' to give suitable reaction rates at pH7·5 in triethanolamine hydrochloride–NaOH buffer. The activity of each was then determined at 25° in 0·1M-phosphate buffer (pH range 5·82–7·83), 50mM-boric acid–NaOH buffer (pH range 8·06–10·26) and 0·1M-triethanolamine hydrochloride–NaOH buffer (pH range 6·95–8·51) against fructose 1,6-diphosphate adjusted to the pH of the assay.

For the pH–stability studies identical dilutions of each enzyme were prepared in the following: (i) phosphate buffer, pH7·5; (ii) triethanolamine hydrochloride–NaOH buffer, pH7·5; (iii) boric acid–borax buffer, pH8·0; (iv) borate–NaOH buffer, pH10; (v) phosphate–citrate buffer, pH4·5; (vi) phosphate–citrate buffer, pH7·5; (vii) acetic acid–sodium acetate buffer, pH4·4. These solutions were assayed for aldolase activity in triethanolamine hydrochloride–NaOH buffer, pH7·5, immediately after preparation and after various time-intervals. The concentration of triethanolamine hydrochloride–NaOH buffer (70mM) in the assay media was sufficient to overcome the final molarity of the buffers used in the stability experiments (5–12·6mM). Between assays the enzyme dilutions were stored at 0° in tubes sealed with Parafilm.

Assay of aldolase activity. Determinations of aldolase activity were made at 25° in a Unicam SP.800 recording spectrophotometer fitted with a constant-temperature cell housing by following the oxidation of NADH at 340nm.

The assay mixture (final volume 1·0ml.) contained (final concentrations): fructose 1,6-diphosphate, pH7·5 (5·0mM), NADH (0·5mM), glycerol 3-phosphate dehydrogenase (0·002unit), triose phosphate isomerase (0·01unit), triethanolamine hydrochloride–NaOH buffer, pH7·5 (70mM), and enzyme, diluted where necessary in 0·1M-phosphate buffer, pH7·5, to give a change of less than 0·1 E_{340} unit/min. The reaction was started by the addition of fructose 1,6-diphosphate after preincubation for 5min. Rates were rectilinear for at least 10min.

In the presence of excess of glycerol 3-phosphate dehydrogenase and triose phosphate isomerase it was assumed that the oxidation of 2μmoles of NADH (E_{340} 6·22) was equivalent to the cleavage of 1μmole of fructose 1,6-diphosphate. The unit of enzyme activity is defined as the cleavage of 1μmole of substrate/min. at 25° and specific activity is defined as units of enzyme activity/mg. of protein.

Blank determinations were performed in the absence of substrate and also in the absence of enzyme and presence of substrate, but detectable activity was not found.

Determination of protein. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Miller (1959), with crystalline egg albumin or bovine plasma albumin as standard.

Buffers. All buffer solutions were prepared in glass-distilled water. pH measurements were made with a Radiometer titrator (type TTT1c) and scale expander (type PHA603Ta) equipped with a glass electrode (type G2222C) (Radiometer, Copenhagen, Denmark). Standardization was carried out against Radiometer pH standard solution (pH6·50±0·02 at 20°).

0·1M-Glycine–NaOH buffer was prepared by adjusting 0·1M-glycine to pH8·25 or pH8·50 with M-NaOH. 126mM-Acetic acid–74mM-sodium acetate buffer, pH4·4, 50mM-boric acid–borax buffer, pH8·0, 50mM-borate–NaOH buffer, pH10·0, 91mM-Na_2HPO_4–54·5mM-citric acid buffer, pH4·5, 185mM-Na_2HPO_4–8mM-citric acid buffer, pH7·5, 0·1M-Na_2HPO_4–NaH_2PO_4 buffer, pH7·5, and 0·1M-triethanolamine hydrochloride–NaOH buffer, pH7·5, were prepared as described in Dawson, Elliott, Elliott & Jones (1959). 10mM-Tris–0·5mM-fructose 1,6-diphosphate–1mM-EDTA–8·4mM-HCl buffer, pH7·35, at 2° was prepared as described by Penhoet *et al.* (1966) and is referred to throughout this paper as 'tris buffer'.

The buffer used for starch-gel electrophoresis was citrate–phosphate buffer, pH7·03, of composition: (*a*) gel buffer, 1·8mM-citric acid–13mM-Na_2HPO_4; (*b*) electrode buffer, 6mM-citric acid–48mM-Na_2HPO_4 (D. N. Baron & C. J. Foxwell, personal communication).

Reagents. Fructose 1,6-diphosphate (trisodium salt), NAD^+ and NADH (disodium salts), triethanolamine hydrochloride, glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) (40units/mg.), triose phosphate isomerase (EC 5.3.1.1) (2400units/mg.) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (36units/mg.) were bought from Boehringer Corp. (London) Ltd., London W.5.

Fructose 1-phosphate (sodium salt) was a gift from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, which is acknowledged with gratitude.

Tris was purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, and 'starch-hydrolysed' was from Connaught Medical Research Laboratories, Toronto, Ont., Canada.

Naphthalene Black 10B (Amido Black 10B) was obtained from George T. Gurr, London S.W.6.

All other chemicals were of the highest purity available from British Drug Houses Ltd., Poole, Dorset.

RESULTS

Distribution of aldolase. The results from homogenization of guinea-pig cerebral cortex in 0·1M-glycine–sodium hydroxide buffer, pH8·5, and 0·32M-sucrose, pH7·0, followed by centrifugation are presented in Table 1. Almost 80% of the total aldolase activity was extracted by 0·1M-glycine–sodium hydroxide buffer (70·7% was recovered in supernatant 1 and a further 8·0% in supernatant 2). Homogenization in 0·32M-sucrose resulted in 57·2% of the aldolase activity being recovered in supernatants 1 and 2. The aldolase activity remaining in the sucrose pellet (42·9%) was twice

Table 1. *Distribution of aldolase between soluble and sedimented fractions prepared in* 0·1M-*glycine–sodium hydroxide*, pH8·5, *and* 0·32M-*sucrose*, pH7·0

Each value is the mean from two preparations. The values in parentheses represent the percentages of total activity recovered.

Homogenization medium	Aldolase activity (μmoles/min./g. of tissue)	
	0·1M-Glycine–NaOH	0·32M-Sucrose
Supernatant 1	4·23 (70·7%)	2·45 (44·5%)
Supernatant 2	0·48 (8·0%)	0·70 (12·7%)
Pellet	1·28 (21·4%)	2·36 (42·9%)

that in the glycine–sodium hydroxide pellet (21·4%). Glycine–sodium hydroxide buffer was therefore the homogenization medium used for the larger-scale preparation of the aldolase as most of the enzyme was obtained in a soluble form in that buffer.

Ammonium sulphate fractionation. The overall recovery of aldolase was 80% (four experiments) in the post-dialysis fractions after ammonium sulphate fractionation; of this total those fractions (0·500–0·650 saturation) that had a specific activity greater than the starting material accounted for 70% of the recovery (Table 2). In the fractions most highly enriched in aldolase (which were precipitated consistently at 0·575–0·600 saturation) increases in the specific activity of three- to four-fold were obtained. Starch-gel electrophoresis showed all five forms of aldolase to be present in all active fractions combined and used subsequently for column chromatography. For this the fractions that were precipitated between 0·500 and 0·650 saturation were combined.

Table 2. *Ammonium sulphate fractionation of guinea-pig cerebral-cortex aldolase*

Each value is the mean of four preparations.

Fraction	Aldolase activity: Total activity (μmoles/min.)	Aldolase activity: Sp. activity (μmole/min./mg. of protein)	Aldolase activity: Recovery (% of original)
Original tissue supernatant	86·0	0·15	(100)
$(NH_4)_2SO_4$			
0–0·500 satn.	8·4	0·04	9·5
0·500–0·575 satn.	17·5	0·31	20·3
0·575–0·600 satn.	25·6	0·61	29·6
0·600–0·650 satn.	16·0	0·40	18·5
0·650–0·675 satn.	1·4	0·16	1·6

DEAE-cellulose chromatography. The first peak of aldolase activity, eluted by 'tris buffer', was not retained by the DEAE-cellulose and contained the largest amount of aldolase activity in any of the five peaks. Four further peaks of aldolase activity were eluted from the column by 40mM-, 50mM- or 55mM-, 0·11M- and 0·14M-sodium chloride in 'tris buffer', which were the minimum molarities of sodium chloride that had been found during preliminary runs to be effective in eluting aldolase activities from the column. Starch-gel electrophoresis (Plate 1) of each peak of aldolase activity followed by staining for enzyme activity and protein indicated that each peak was composed of only one enzyme type and that contamination of any type by others had not occurred. Only one region of protein stain was found to correspond to each region of enzyme stain. All isoenzyme types present in the original extract were recovered from the column.

The mean overall recovery from three DEAE-cellulose columns was 85·7%. The contribution of each isoenzyme to this total was: V, 33·3%, IV,

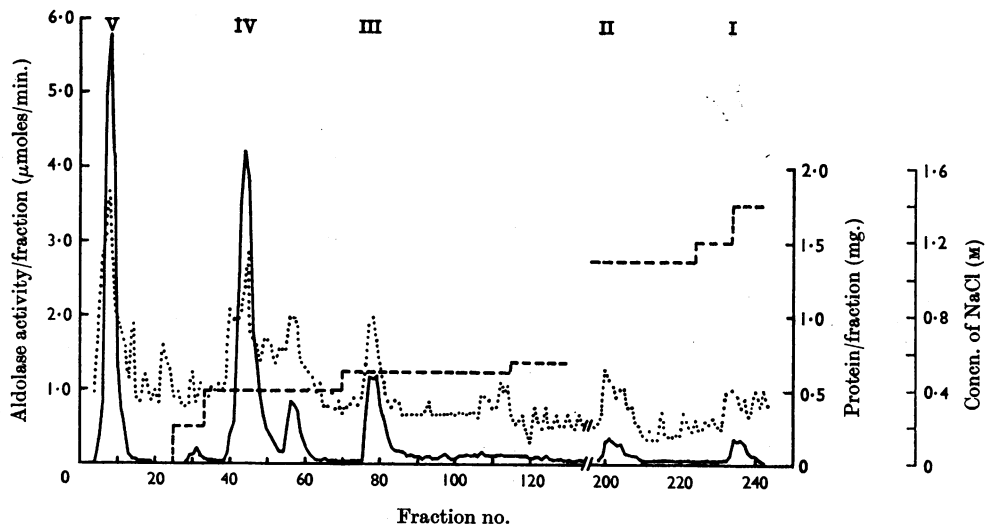
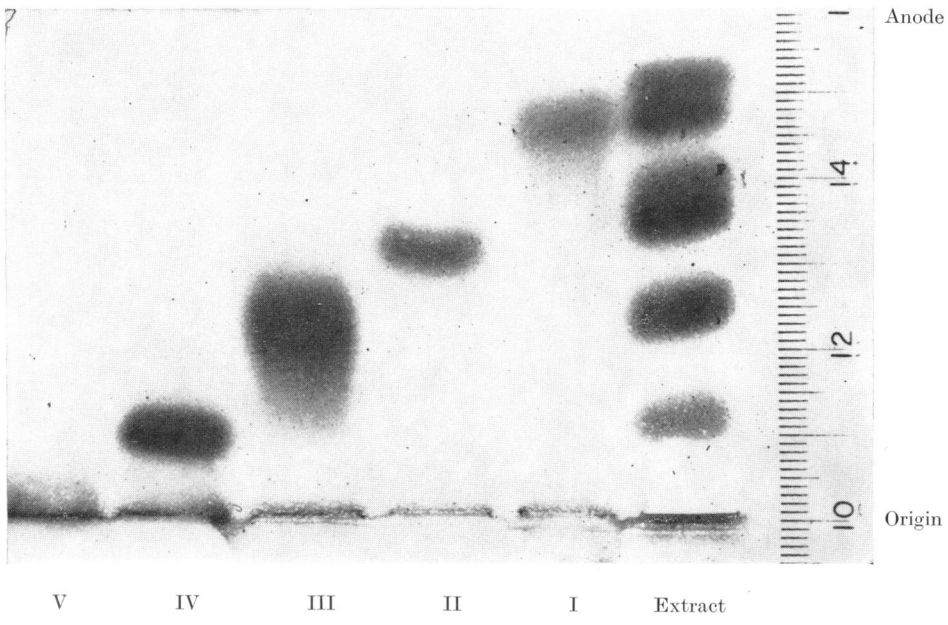


Fig. 1. DEAE-cellulose chromatography of aldolase precipitated between 0·500 and 0·650 saturation with $(NH_4)_2SO_4$ as described in the Materials and Methods section. Peaks are numbered (V–I) according to aldolase isoenzyme content (see the introduction and Plate 1). ——, Aldolase activity; ....., protein; ----, concn. of NaCl in the eluting buffer.



EXPLANATION OF PLATE 1

Starch-gel electrophoresis in citrate–phosphate buffer, pH 7·03, of aldolase isoenzymes separated by DEAE-cellulose chromatography. Extract: original supernatant from guinea-pig cerebral cortex homogenized in glycine–NaOH buffer as described in the Materials and Methods section. I–V: peaks eluted from DEAE-cellulose column chromatography (Fig. 1) as described in the text.

25·7%; III, 15·3%; II, 5·2%; I, 6·2%. The specific activities of each isoenzyme varied from 0·1 in the first fraction of each eluted peak to 3·2 (V), 3·5 (IV), 4·6 (III), 1·2 (II) and 0·7 (I) in the most active fractions of each type. The elution pattern is shown in Fig. 1.

Effect of pH *on activity.* The effects of variation of pH on the activity of the five isoenzymes were examined in various buffers as described in the Materials and Methods section in the presence of excess of glycerol 3-phosphate dehydrogenase (0·11 unit/1·0 ml. assay volume) and triose phosphate isomerase (0·67 unit/1·0 ml. assay volume). Bell-shaped pH–activity curves were obtained for all five isoenzymes in phosphate and borate buffers with pH optima in the range 7·5–8·0 (Fig. 2). When assayed in triethanolamine hydrochloride–sodium hydroxide buffer the five isoenzymes all gave higher activities than in phosphate and borate buffers (Table 3) and presented flat pH–activity profiles in the range 6·95–8·51 (Fig. 2).

Effect of pH *on stability.* All the cerebral aldolase isoenzymes were completely stable in phosphate buffer, pH 7·5, for 26 days and with the exception of isoenzyme V were slightly more active at the end of the stability experiments than at the beginning. In triethanolamine hydrochloride–sodium hydroxide buffer, pH 7·5, and in borate buffers at pH 8·0 and pH 10·0 all forms lost very little activity (1–2%/day) with the exception of isoenzyme I, which showed no loss of activity at pH 10·0.

The most striking differences in stability were observed on storage at pH 4·5 (Fig. 3). Isoenzyme V lost its activity within 2 days at 0°; isoenzyme IV was less unstable with a loss of activity of 54% in 4 days and over 90% in 2 weeks. Isoenzyme III was more stable than isoenzyme IV or isoenzyme V, losing 54% of its activity in 14 days. Isoenzymes I and II were stable at pH 4·5 for at least 26 days after an initial small decrease in activity. That the instability observed was due to the pH rather than to the buffer constituents was indicated from a comparison of the stabilities of isoenzymes IV and V in phosphate–citrate buffer, pH 7·5, and acetate buffer, pH 4·4, with phosphate–citrate buffer, pH 4·5 (Fig. 4). The rate of loss of activity of isoenzyme V was similar in acetate buffer, pH 4·4, or phosphate–citrate buffer, pH 4·5 (a loss of 50%

Table 3. *Activities of aldolase isoenzymes at* pH 7·5–8·0 *in phosphate, borate and triethanolamine hydrochloride–sodium hydroxide buffers*

	Aldolase activity (nmoles/min.)		
Isoenzyme	Phosphate	Borate	Triethanolamine hydrochloride–NaOH
I	6·1	6·1	7·4
II	3·1	3·7	4·0
III	6·4	6·6	8·2
IV	7·3	7·8	10·6
V	6·0	5·0	7·85

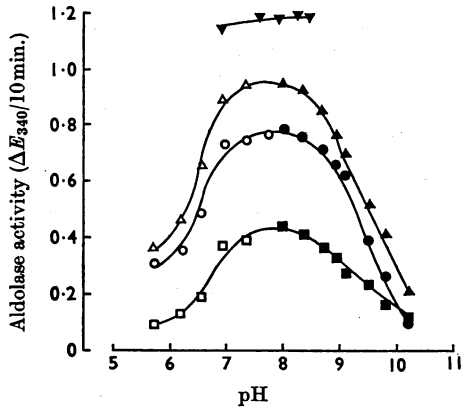


Fig. 2. pH–activity curves for aldolase isoenzymes. ○ and ●, Isoenzyme I; □ and ■, isoenzyme II; △ and ▲, isoenzyme IV in phosphate buffer, pH 5·82–7·83, and borate buffer, pH 8·18–10·26; ▼, isoenzyme IV in tri-ethanolamine hydrochloride–NaOH buffer, pH 6·95–8·51.

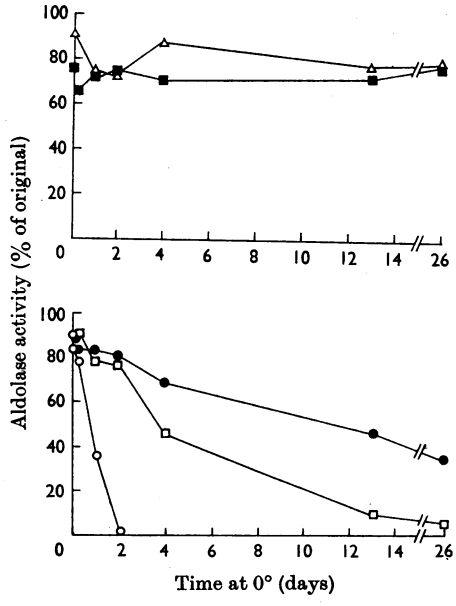


Fig. 3. Stability of aldolase isoenzymes at pH 4·4 in phosphate–citrate buffer, as described in the text. △, Isoenzyme I; ■, isoenzyme II; ●, isoenzyme III; □, isoenzyme IV; ○, isoenzyme V.

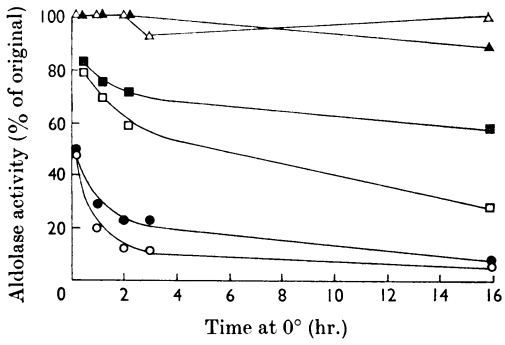


Fig. 4. Stabilities of aldolase isoenzymes IV and V at pH4·5 (acetate buffer), pH4·4 and 7·5 (phosphate–citrate buffer) as described in the text. △, Isoenzyme V, phosphate–citrate buffer, pH7·5; ▲, isoenzyme IV, phosphate–citrate buffer, pH7·5; ■, isoenzyme IV, acetate buffer, pH4·5; □, isoenzyme IV, phosphate–citrate buffer, pH4·4; ●, isoenzyme V, acetate buffer, pH4·5; ○, isoenzyme V, phosphate–citrate buffer, pH4·4.

within the first 9min.), but the enzyme was stable in phosphate–citrate buffer, pH7·5. Similar results were observed for isoenzyme IV, in that the enzyme was stable at pH7·5 but less so in the mildly acidic buffers.

The activity that was lost in isoenzymes IV and V after storage at pH4·4 or 4·5 was not recovered by dialysis of the inactivated enzyme preparations against 0·1M-phosphate buffer, pH7·5, for 4hr. at 0°.

Substrate activity ratios. The substrate activity ratios of each form of aldolase were compared with fructose 1,6-diphosphate (5mM) and fructose 1-phosphate (5mM) at pH7·5 in triethanolamine hydrochloride–sodium hydroxide buffer. The fructose 1,6-diphosphate/fructose 1-phosphate activity ratio was highest for isoenzyme III and lowest for isoenzymes I and V. The value for each isoenzyme was: I, 12; II, 24; III, 44; IV, 25; V, 19.

DISCUSSION

The experiments carried out to determine the distribution of brain aldolase between soluble and particulate fractions indicated clearly that the proportion obtained in the soluble fraction depended on the medium used. With glycine–sodium hydroxide buffer 80% of the total brain aldolase activity appeared in the soluble fraction, in contrast with 57% with sucrose. The remainder of the activity appeared to be firmly associated with the sedimented fractions.

The ammonium sulphate fractionation of the enzyme in buffered solutions resulted in the removal, in the fractions from 0 to 0·500 saturation, of 45% of the protein with a very low aldolase content of specific activity 0·04μmole/min./mg. of protein. Subsequent fractions, especially those that were precipitated at 0·500–0·600 saturation, had three- to four-fold higher specific activities. The total recovery was 80%.

DEAE-cellulose chromatography and stepwise elution with a sodium chloride–'tris buffer' gradient led to the complete separation of multiple forms of aldolase in almost quantitative yield and with substantially increased specific activities in the most active fractions. The electrophoretically distinct isoenzymes are considered to be separate molecular species on the basis of elution characteristics from the DEAE-cellulose and differences in pH–stability curves and substrate activity ratios. The relatively small amounts of enzyme prepared so far have prevented extensive investigations of the purity of the isoenzymes by other methods, but enzyme and protein staining after electrophoretic separation did indicate enzymic and protein homogeneity.

The pH optima values of 7·5–8·0 as determined in phosphate and borate buffers were all similar and resemble the value of pH7·5–8·0 reported for rabbit liver aldolase (Rajkumar, Woodfin & Rutter, 1966). Peanasky & Lardy (1958*a*) found, however, the optimum pH for cleavage of fructose 1,6-diphosphate by bovine liver aldolase in the presence of CN^- ion to be pH9·1–9·4. The broad pH optima of brain aldolase enzymes in triethanolamine hydrochloride–sodium hydroxide buffer resemble that of rabbit muscle aldolase, pH6·5–9·0 determined by aldol synthesis (Richards & Rutter, 1961). It is possible that the observed pH optimum of aldolase may be greatly dependent on the buffer and method of assay used.

Values for pK_a and pK_b were calculated from the pH values at which the velocity was half-maximal by using the equations of Alberty & Massey (1954). The pK_a values for guinea-pig cerebral-cortex aldolase (all forms) were in the range 6·31–6·55 and the pK_b values were in the range 9·45–9·59. The pK_a values lie in the range given for the imidazolium group (5·6–7·0) and the pK_b values in the range for the ϵ-amino group of lysine (9·4–10·6) (Dixon & Webb, 1964). Evidence has been presented to indicate the involvement of lysine at the active centre of rabbit muscle aldolase (Horecker *et al.* 1963) and also that histidine may be involved (Hoffee, Lai, Pugh & Horecker, 1967).

The difference in the shapes of the pH–activity curves determined in triethanolamine hydrochloride–sodium hydroxide buffer and in phosphate and borate buffers is of interest. The activity of triose phosphate isomerase is not pH-dependent in the range pH7·0–8·0, but is decreased by 50% at

pH 6·3 (Oesper & Meyerhof, 1950) and, in addition, activity is decreased by 75% in the presence of 50mM-phosphate (Topper, 1961). Consequently in pH–activity assays the glycerol 3-phosphate dehydrogenase–triose phosphate isomerase coupling system was added in excess and the maximal aldolase rate at pH 7·5–8·0 did not exceed the capacity, at pH 6·3 and in the presence of 50mM-phosphate, of the coupling enzymes. It therefore seems unlikely that the observed effects of pH, in phosphate and borate buffers, were due to effects on the coupling enzymes rather than on the aldolase.

Hartman & Barker (1965) investigated the active centre of rabbit muscle aldolase with structural analogues of fructose 1,6-diphosphate and concluded that the binding of fructose 1,6-diphosphate was due primarily to the phosphate groups. Ginsberg & Mehler (1966) studied specific anion binding to rabbit muscle aldolase and demonstrated two highly specific binding sites for phosphate at or near the active centre and, from the magnitudes of the affinity constants, postulated co-operating positive charge clusters at the active centre.

These observations lend support to a possible explanation for the higher activities noted in triethanolamine hydrochloride–sodium hydroxide buffer, that phosphate and borate ions are occupying specific sites on the aldolase molecule and these impede the approach or binding of fructose 1,6-diphosphate to the active site. In triethanolamine hydrochloride–sodium hydroxide buffer in the absence of phosphate and borate ions there was little change in the maximum rate in the range pH 6·95–8·51 and reaction rates were faster than in phosphate or borate buffer at similar enzyme concentrations.

The substrate activity ratios (12–44) found for the individual aldolase isoenzymes are in good agreement with the value of 26 for rabbit brain aldolase (Blostein & Rutter, 1963) and are closer to the value reported for rabbit muscle aldolase (57) than for rabbit liver aldolase (1·2) (Blostein & Rutter, 1963). Rensing *et al.* (1967) determined substrate activity ratios for bovine brain aldolases to be 8·1, 10·9, 12·0, 13·8 and 25·5 (isoenzymes I–V), which contrasts with the present finding for guinea-pig that isoenzyme III has the highest ratio. The substrate activity ratios given by Foxwell *et al.* (1966) for rabbit brain aldolase isoenzymes are, in general, considerably higher (26–167)

The high substrate activity ratios of brain and muscle appear to reflect the fact that fructose 1-phosphate is not a normal metabolite in these tissues, whereas liver aldolase has a specific role in metabolizing dietary fructose 1-phosphate (Spolter, Adelman, Di Pietro & Weinhouse, 1965). Under physiological conditions therefore it seems likely that the only natural substrate for brain aldolases is fructose 1,6-diphosphate.

Penhoet *et al.* (1966, 1967) isolated five isoenzymes of aldolase from rabbit brain and resolved A ('muscle type') and C ('brain type') subunits by electrophoresis after dissociation of the isoenzymes at pH 2. After recombination at pH 7·5, 75% of the original enzymic activity was recovered. They suggested that each aldolase isoenzyme was a tetramer of A and C subunits, giving five isoenzymes: I, C_4; II, C_3A; III, C_2A_2; IV, CA_3; V, A_4. Rensing *et al.* (1967) were able to reassociate bovine or rabbit A-type subunits with bovine C-type subunits to give five hybrid forms, using similar procedures.

If it is assumed that the guinea-pig cerebral-cortex aldolase isoenzymes are similarly constituted, then the gradual increase in stability in phosphate–citrate buffer, pH 4·5, and acetic acid–acetate buffer, pH 4·4 (isoenzyme V < IV < III < II and I), correlates well with the change in character of the constituent subunits in each form. The marked instability under acidic conditions of isoenzymes IV and V would be related to the preponderance of A subunits in these molecules. Increasing the number of C subunits in isoenzymes III, II and I would increase the stability to acidic conditions. However, the stability of isoenzymes V, IV and III in phosphate buffer, pH 7·5, implies that, if inactivation is caused by dissociation, phosphate ions may prevent the dissociation by interaction with the A subunits.

The inactivation of isoenzymes IV and V in two different buffers at pH 4·4 and pH 4·5 suggests that the effect is due to pH and not to the ionic constituents of the buffers.

The inability to recover enzymic activity at pH 7·5 after inactivation of isoenzymes IV and V at pH 4·4 or 4·5 is in contrast with the reversible dissociation reported by Penhoet *et al.* (1966, 1967). It would appear that, if the subunit content of guinea-pig brain aldolases is similar to those found in the brains of other species, the A_4 and AC_3 conformations in guinea-pig aldolases may be particularly unstable and the dissociation is not readily reversed by a return to the original conditions.

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