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A Comparative Study of Aldolase from Human Muscle and Liver

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Aldolase was purified from human skeletal muscle and human liver by techniques capable of processing large quantities (10–20kg) of tissue. The methods used also proved convenient for isolating aldolase on a large scale from other mammalian and avian sources. Aldolase from both human liver and muscle was crystallized; each gave two crystalline forms, depending on the conditions of crystallization. X-ray studies on the muscle aldolase crystals suggest a close structural similarity between human and rabbit muscle aldolase. Aldolases from human muscle and liver have similar pH optima and pH stability but their stability to heat treatment differs. The effect of heat on the enzymes may therefore provide an easy means of distinguishing them. The kinetic constants K_m and $k_{cat.}$ for these aldolases are similar to other mammalian aldolases. Amino acid analyses and tryptic peptide 'mapping' show that the primary structures of the two aldolases differ greatly.

In mammalian tissues aldolase (EC 4.1.2.13) exists in three primary forms; aldolase A predominates in muscle, B in liver and kidney and C, with a variety of hybrids, in brain. Rutter and co-workers have identified these forms on the basis of antigenic specificity, catalytic properties and electrophoretic mobility (Penhoet *et al.*, 1966; Lebherz & Rutter, 1969).

Rabbit muscle aldolase has been studied extensively. It has mol.wt. 158000 (Kawahara & Tanford, 1966; Sia & Horecker, 1968) and consists of four subunits that are nearly identical (Penhoet *et al.*, 1967). Sequence studies on this enzyme have been started and cleavage at the 3 methionine residues per subunit with cyanogen bromide (Lai & Chen, 1968) together with tryptic digestion (Lai, 1968) has permitted 110 out of a total of about 364 amino acids to be located.

Aldolase has also been crystallized and studied from a number of other mammalian muscles, rat (Matsushima *et al.*, 1968; Gracy *et al.*, 1970), ox (Anderson *et al.*, 1969; Domagk *et al.*, 1971) and pig (Anderson *et al.*, 1969; Gibbons *et al.*, 1970), and from non-mammalian sources, e.g. sturgeon (Anderson *et al.*, 1969), chicken (Marquardt, 1969) and frog (Ting *et al.*, 1971). Considerable homology exists between the sequences of muscle aldolase isolated from a variety of mammalian and non-mammalian sources (Anderson *et al.*, 1969). In the sequence of the tryptic peptide around the active-site lysine of aldolase from the muscle of rabbit, codfish, sturgeon, frog and lobster, 12 residues are invariant and only 8 residues are substituted from a total of 28 in this region.

Aldolase B has been isolated from the liver of rabbit (Gracy *et al.*, 1969), ox (Peanasky & Lardy, 1958), rat (Gracy *et al.*, 1970) and man (Gürtler & Leuthardt, 1969). The enzyme from liver has a specific activity of about 1–1.5 units/mg, which contrasts with aldolase from muscle, which has an activity in the range 10–20 units/mg. The substrate specificity of these two enzymes is also different: liver aldolase catalyses the cleavage of fructose 1-phosphate and fructose 1,6-diphosphate at approximately equal rates whereas the muscle enzyme cleaves fructose 1-phosphate and fructose 1,6-diphosphate at rates in the ratio of approximately 1:50. Rutter *et al.* (1963) suggested that these differences are due to the different functions of these enzymes; liver aldolase is found where some variation in substrate specificity is useful, e.g. in fructose metabolism in liver, compared with muscle aldolase, which is found in glycolytic tissues. However, the molecular weight and subunit structure of the enzymes from liver and muscle appear to be similar (Gracy *et al.*, 1969; Peanasky & Lardy, 1958) and considerable homology exists in the active-site tryptic peptide of rabbit aldolase isolated from these two sources (Forcina & Perham, 1971).

A transition from aldolase A to aldolase B occurs during the development of rat liver (Rutter *et al.*, 1963). By about 15 days after birth aldolase A, present in the embryo, is almost entirely replaced by aldolase B. A similar change also occurs in the liver of human embryos (Lebherz & Rutter, 1969). Gracy *et al.* (1970) showed that these replacements can occur in the adult animal and Ikehara *et al.* (1970) showed that in the Novikoff tumour in rat liver, the liver aldolase is replaced by muscle aldolase. Changes in the isoenzyme pattern have also been observed in a variety of tumours (Schapira *et al.*, 1963; Nordmann & Schapira, 1967), and the direct assay of serum

aldolase can be used diagnostically to detect not only tumours but many other disorders (Katz & Ducci, 1958; Norberg, 1961). A rise in serum aldolase activity is found when cellular damage occurs and a direct correlation has been observed between the aldolase activity in serum and the presence of leukaemia (Sibley & Fleisher, 1954). An increase in serum aldolase activity has also been observed in patients with primary liver cancer (Schapira, 1962), muscular dystrophy (Sibley & Lehninger, 1949), myocardial infarction (Rowell & Smith, 1959) and in a variety of other disorders.

In this present paper we describe the purification of aldolase from human skeletal muscle and liver, and compare the properties of these enzymes with aldolase isoenzymes isolated from other mammalian species. Studies were also carried out to find a way of distinguishing between these two isoenzymes that could be applied to the clinical determination of aldolase in human serum.

Experimental

Materials

Crystalline fructose 1,6-diphosphate was purchased from Wessex Biochemicals Ltd., Castle Rd., Bournemouth BM9 1PH, U.K. Whatman CM-cellulose and DEAE-cellulose (grades 11 and 32) were obtained from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. and were precycled immediately before use; 5,5′-dithiobis-(2-nitrobenzoic acid) was purchased from R. N. Emanuel Ltd., Wembley, Middx., U.K. In the initial stages of the purification procedure BDH $(NH_4)_2SO_4$ (98% pure) was employed, but during the later stages AnalaR $(NH_4)_2SO_4$ was used for all precipitations. All other chemicals were obtained from either Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., or BDH Chemicals Ltd., Poole, Dorset, U.K., and were the purest obtainable. Triose phosphate isomerase was obtained from chicken muscle by a method developed in this laboratory based on the method of Putman *et al.* (1972). Glycerol 1-phosphate dehydrogenase was purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Rabbit muscle was obtained from Wessex Biochemicals Ltd., Bournemouth, Hants., U.K., and chicken muscle from a local butcher. Human muscle and liver were obtained from the Radcliffe Infirmary, Oxford. Calcium phosphate was prepared by the method of Keilin & Hartree (1938).

Methods

Assay. Throughout the purification procedures for both muscle and liver aldolase, the activity was assayed at each stage by a method based on the coupled-enzyme system of Racker (1947). A 3ml quartz cuvette contained 0.1ml of 60mM-fructose 1,6-diphosphate, 0.1ml of 0.15mM-NADH, 5μl of glycerol 1-phosphate dehydrogenase (5 mg/ml), 2μl of triose phosphate isomerase (2mg/ml) and 2.7ml of 0.1M-Tris–5mM-EDTA adjusted to pH7.5 or 7.2 with 1M-HCl. The decrease in E_{340} on the addition of 50μl of the sample was monitored on a Pye Unicam SP.1800 spectrophotometer. One unit of aldolase activity was defined as the amount catalysing the cleavage of 1μmol of fructose 1,6-diphosphate/min at 25°C.

Protein determination. During the initial stages of purification protein was determined by the method of Lowry *et al.* (1951) by using bovine serum albumin (containing <1.5% water, w/w) as standard. A 1% solution of purified muscle aldolase in water was determined to have an extinction $E_{1cm}^{1\%} = 9.2$ and liver aldolase $E_{1cm}^{1\%} = 8.5$.

Electrophoresis. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was performed by the method of Weber & Osborn (1958). Cellulose acetate electrophoresis was done on Gelman Seprophore III paper. The enzyme was always exhaustively dialysed before use against the same buffer that was used for electrophoresis. Protein was stained with Ponceau S and destained with 1% acetic acid.

General methods used in the preparations. Because the precipitation behaviour of enzymes depends considerably on the method of addition of $(NH_4)_2SO_4$, all precipitations were done as follows. (*a*) Solid $(NH_4)_2SO_4$ was added with vigorous stirring over a 2–5min period. (*b*) Stirring was continued for a further 10min and the solution was then left to equilibrate for at least 30min. (*c*) All operations were done between 4 and 8°C.

Precipitates were either removed by filtration where the volumes were large or by centrifugation on MSE 18 and MSE 6L centrifuges. Dialysis was performed at 4°C in a constant-shaking device of 60 litres capacity.

Preparation of human muscle aldolase. Muscle was obtained *post mortem* and stored frozen at −20°C until sufficient was obtained for a preparation. Initially, small batches (1kg) were processed and these gave final specific activities in the range 7–8 units/mg. Repeated column chromatography and $(NH_4)_2SO_4$ fractionation failed to increase this value. The enzyme was only obtained in a pure form, however, when larger batches were processed (5–7kg). During the study 10 batches of the enzyme were prepared and it was apparent that the muscle contained little aldolase activity. The average specific activity of the initial extract for these 10 batches was 0.3 unit/mg; the total extractable activity per g wet weight of muscle was 13.2 units and the total protein per g wet weight of muscle was 44mg. Comparable figures from the extraction of rabbit muscle aldolase by using fresh muscles are 3 units/mg, 80 units, and

40mg. The low yield of aldolase that was extracted from human muscle was probably due to the age of the tissue that was used together with the effects of autolysis.

The purification procedure was as follows. (1) The soluble muscle enzymes were extracted by blending with 3–4 times the volume of cold 1mM-Tris–5mM-EDTA–1mM-mercaptoethanol adjusted to pH7.5 with 1M-HCl in a Waring Blendor (4.5 litres capacity) for 1min. The extract was then adjusted to pH5.4 with 1M-acetic acid.

(2) The debris was spun down in an MSE Mistral 6L centrifuge at 2000 rev./min for 20min. at 5°C and discarded. The supernatant was filtered through washed glass-wool to remove fat-particles and the pH was adjusted to 7.4 with 1M-NaOH.

(3) $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation (24.3g/100ml) at 4°C and the precipitate was centrifuged down and discarded. The solution was then adjusted to 60% saturation with $(NH_4)_2SO_4$ (13.2g/100ml) and the precipitate was collected by filtration under vacuum on Whatman 52 filter paper at 4°C. The dry precipitate was removed and dialysed against 5mM-Tris–HCl–1mM-EDTA, pH7.5.

(4) The dialysis residue was clarified by centrifugation and then applied to a column (9cm×150cm) of DEAE-cellulose 11 previously equilibrated with the same buffer. The column was washed with this buffer to elute aldolase, which was not retained, and fractions with the highest specific activity were combined.

(5) Calcium phosphate, equilibrated with 5mM-Tris–HCl–1mM-EDTA, pH7.5, was added to the combined fractions, to give a concentration of about 0.5mg/mg of protein and stirred at room temperature for 30min, then the calcium phosphate was removed by centrifugation.

(6) The supernatant contained all the activity and $(NH_4)_2SO_4$ was added to 60% saturation (39g/100ml). The precipitate was collected by filtration on Whatman 52 filter paper at 4°C. The dried precipitate was collected and dialysed free of salt against 5mM-Tris–HCl–1 mM-EDTA, pH7.5. The dialysis residue was then adjusted to pH6.5 with 0.2M-acetic acid and applied to a column (9cm×150cm) of CM-cellulose 11 that had been equilibrated with 5mM-Tris–1mM-EDTA–1mM-mercaptoethanol adjusted to pH6.5 with 1M-HCl.

(7) The column was washed with buffer until all the non-absorbed protein had passed through and then a linear gradient of 0–0.2M-NaCl of 3 litres capacity, in the same buffer, was applied. Fractions with a specific activity greater than 10 units/mg were pooled and adjusted to 60% saturation with $(NH_4)_2SO_4$ (39g/100ml) as before.

(8) The precipitate was collected by centrifugation, dialysed and applied to a column (3.5cm×60cm) of CM-cellulose 52 by using similar conditions to those used in stage (6). The aldolase was eluted with a linear gradient of 0–0.2M-NaCl and fractions with the highest specific activities were pooled.

Purification of aldolase from human liver. Liver was stored frozen at −20°C until use and batches (5kg or greater) were processed as follows.

(1) Aldolase was extracted by blending the liver in 1mM-Tris–HCl–1mM-EDTA–5mM-mercaptoethanol, pH7.5, and the debris was removed by centrifugation.

(2)The supernatant was adjusted to pH5.4 with 1M-acetic acid and the precipitate removed by centrifugation. The clear supernatant was readjusted to pH7.5 and $(NH_4)_2SO_4$ added to give 40% saturation (24.3g/100ml). The precipitate was removed by centrifugation and discarded. $(NH_4)_2SO_4$ was then added to give 60% saturation (13.2g/100ml) and the precipitate, which contained all the aldolase activity, was collected by filtration on Whatman 52 filter paper in the cold.

(3) The precipitate was dialysed free of salt against 5mM-Tris – HCl – 1mM-EDTA – 1mM-mercaptoethanol, pH7.5, and the dialysis residue was applied to a column (9cm×150cm) of DEAE-cellulose 11 equilibrated with the same buffer. On washing the column with the same buffer, aldolase passed through the column and fractions with a specific activity greater than 0.2 unit/mg were pooled and precipitated to 60% saturation (39g/100ml) with $(NH_4)_2SO_4$ in the cold.

(4) The precipitate was centrifuged and dialysed against 5mM-Tris–HCl–1mM-EDTA–1mM-mercaptoethanol, pH7.5. The dialysis residue was adjusted to pH6.5 with 0.2M-acetic acid and clarified by centrifugation. This was then applied to a column (9cm×150cm) of CM-cellulose 11 equilibrated with 5 mM - Tris – HCl – 1mM-EDTA – 1mM-mercaptoethanol, pH6.5, and was washed until no protein appeared in the washings. Occasionally some aldolase activity was found in these washings although most of the activity stayed on the CM-cellulose at this stage. The aldolase was eluted with a linear gradient of 0–0.3M-NaCl of 3 litres capacity, in the same buffer.

(5) This fraction was precipitated with $(NH_4)_2SO_4$ and the precipitate dialysed free of salt in a similar manner to that described above and applied to a column (3.5cm×60cm) of CM-cellulose 52. The aldolase was eluted with a linear gradient of 0–0.3M-NaCl of 2 litres capacity and fractions with a specific activity of greater than 1.1 units/mg were combined and precipitated to 60% saturation with $(NH_4)_2SO_4$ (39g/100ml).

Preparation of aldolase from rabbit and chicken muscle. Aldolase from rabbit and from chicken muscle was prepared by methods similar to those used for human muscle aldolase. The specific activity of both of these enzymes was between 12 and 15 units/mg

and they were pure as judged by polyacrylamide-gel electrophoresis.

Purity of enzymes used in this study. Kinetic studies, amino acid analyses and tryptic peptide 'mapping' were done on samples of muscle enzyme that had a specific activity of 16.8 units/mg. All the studies on liver aldolase were performed on protein with a specific activity of 1.1 units/mg. Unless otherwise stated the liver was always dialysed against buffer containing 0.5mM-mercaptoethanol. If this reagent was omitted during dialysis considerable precipitation of the enzyme occurred.

Carboxymethylation of aldolase from human muscle and liver. For the amino acid analyses and the tryptic peptide 'mapping' the enzymes were carboxymethylated in the following manner. Protein (5–10mg/ml) was dialysed overnight against 0.1M-Tris buffer, adjusted to pH8.6 with HCl and containing 8M-urea and 20mM-mercaptoethanol. Iodoacetate, freshly recrystallized from petroleum ether (b.p. 60–80°C) was then added to give an 8-fold excess over the thiol groups. The mixture was incubated for 4h at 37°C. The carboxymethylated protein was then dialysed against 0.5% NH_4HCO_3 at room temperature and stored as a freeze-dried powder.

Amino acid analysis. Samples (1mg) of carboxymethylated enzyme were dissolved in Aristar 6M-HCl in acid-washed tubes. The tubes were flushed three times with N_2 and then sealed under vacuum. The samples were incubated at 105°C for 24, 48 and 72h. After incubation the samples were dried under vacuum and analysed by using a Beckman 120C automatic analyser and a Locarte amino acid analyser.

The total thiol content was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) by the method of Ellman (1959). The reaction mixture consisted of 0.1M - Tris – 1mM - 5,5' - dithiobis - (2 - nitrobenzoic acid)–0.5% sodium dodecyl sulphate adjusted to pH7.6 with 0.1M-HCl. The change in E_{412} caused by the addition of dialysed protein was measured on a Gilford 240 recording spectrophotometer. The blank contained an equivalent amount of dialysis buffer. The extinction of the liberated 5-thio-2-nitrobenzoate anion under the conditions used was checked by using standard solutions of 2-mercaptoethanol. The ratio of the tyrosine/tryptophan content of the proteins was determined by the spectrophotometric method of Goodwin & Morton (1946). The tryptophan content was then calculated from the tyrosine content established by amino acid analysis.

For both the thiol content and the tyrosine and tryptophan determinations rabbit muscle aldolase prepared by the method of Eagles *et al.* (1969) with a purity of greater than 99% was used as an internal standard. The amino acid analyses were repeated three times on different preparations of the enzyme and the results were averaged.

Tryptic peptide 'mapping'. To 4mg of carboxymethylated enzyme in 0.5% NH_4HCO_3 was added 0.04mg of crystalline trypsin; the samples were incubated for 4h at 37°C before being dried and dissolved in a minimal amount of 0.05M-NH_3. The peptides were spotted on to Whatman chromatography paper no. 1 and electrophoresis was performed at 50V/cm at room temperature in pyridine–acetic acid–water (25:1:225, by vol.), pH6.5. The neutral peptides were cut out and re-run by using the same conditions at pH3.5 in pyridine–acetic acid–water (1:10:89, by vol.). The areas containing the acidic, basic and neutral peptides were then sewn into chromatography paper and chromatographed in butan-1-ol–acetic acid–water–pyridine (30:6:24:20, by vol.). After chromatography the papers were stained with cadmium–ninhydrin acetate.

After 2h of drying the peptides were marked and recorded.

Molecular weight of aldolase from human muscle and liver. Throughout this study it was assumed that the molecular weights of these enzymes were similar to the aldolases isolated from rabbit (Gracy *et al.*, 1969; Sia & Horecker, 1968) and therefore a molecular weight of 158000 was used for aldolase from both liver and muscle in the calculations.

Results

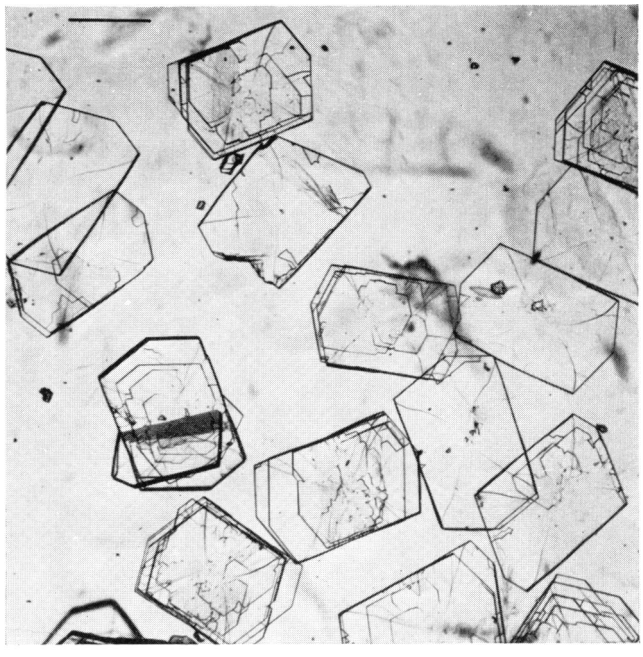
Preparation of human aldolase

The results from a typical preparation of aldolase from human skeletal muscle and human liver are shown in Tables 1 and 2 respectively. The purified enzyme from muscle had a specific activity of 16.8 units/mg. The enzyme gave one band on cellulose acetate electrophoresis and was better than 98% pure as judged by polyacrylamide gels run in the presence of sodium dodecyl sulphate. Purified human liver aldolase had a specific activity of 1.1 units/mg and polyacrylamide-gel electrophoresis showed the enzyme to be more than 97% pure. During the preparation of liver aldolase it was found that a proportion of the sample applied to the CM-cellulose column was not retained and eluted with the front protein peak.

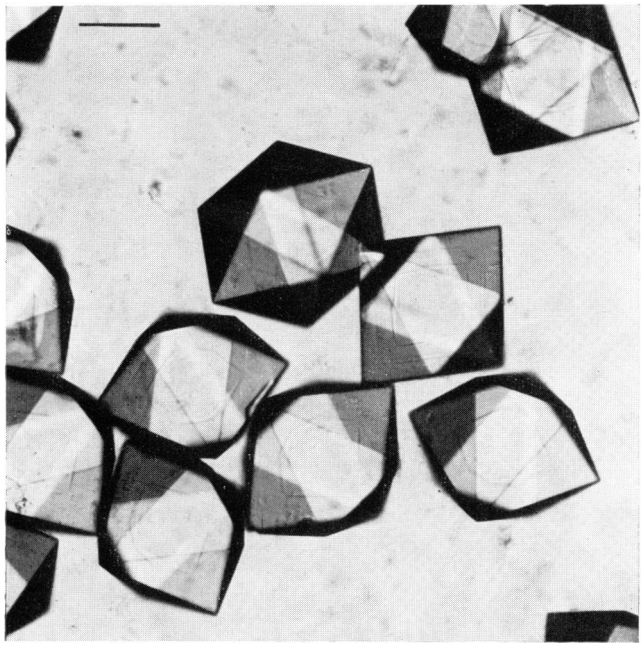
Crystallization of human aldolase and X-ray results

Both the enzymes crystallized in two forms depending on the conditions. At room temperature muscle aldolase crystallized from 0.1M-Tris–HCl–5mM-EDTA, pH7.0, on the addition of $(NH_4)_2SO_4$ to 50% saturation (31.3g/100ml). The largest crystals were formed when the protein concentration was greater than 10mg/ml. These crystals are shown in Plate 1(*a*). At 4°C in the same buffer hexagonal bipyramids could be grown as shown in Plate 1(*b*). The hexagonal crystals were visible after 1 week's growth whereas the plates took longer to grow (3–4

(*a*)



(*b*)

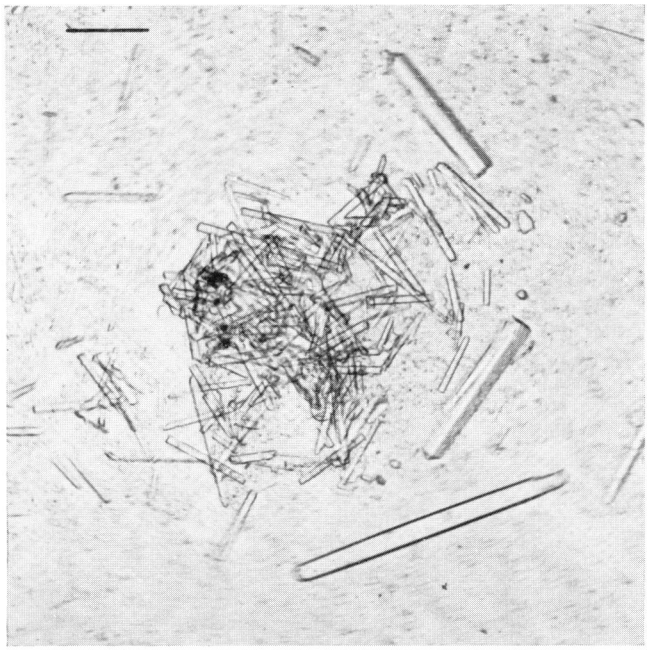


EXPLANATION OF PLATE 1

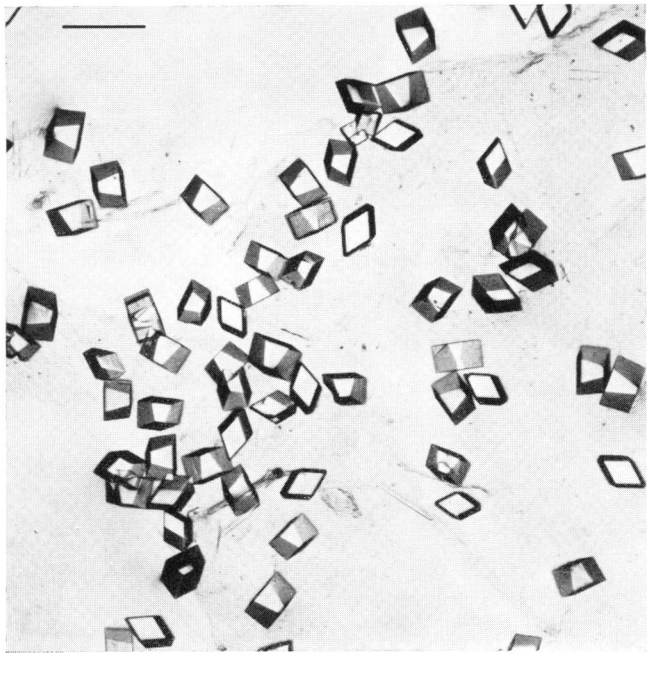
Crystals of aldolase from human muscle

(*a*) Large plate-like crystals of muscle aldolase grown from 0.1 M-Tris–HCl buffer, pH 7.0, at room temperature. (*b*) Hexagonal bipyramid crystals. The bar on the plates represents 0.1 mm.

(*a*)



(*b*)



EXPLANATION OF PLATE 2

Crystals of aldolase from human liver

(*a*) Needles of liver aldolase grown from 0.1M-Tris–HCl buffer containing 2mM-mercaptoethanol at pH7.0. (*b*) Prisms of liver aldolase grown at 4°C. The bar on the plates represents 0.1mm.

P. A. M. EAGLES AND M. IQBAL

Table 1. *Purification procedure for aldolase from 5kg of human skeletal muscle*

Stage	Volume	Units/ml	Protein (mg/ml)	Total units	Units/mg of protein	% yield
(1) Extraction in 1mM-Tris–5mM-EDTA–1mM-mercaptoethanol, pH7.5	13.1 litres	4.6	13.0	60260	0.35	100
(2) After pH5.4 precipitation	13.1 litres	4.2	10.2	55000	0.41	91
(3) $(NH_4)_2SO_4$ precipitation. Precipitate spun and dialysed against Tris–HCl–EDTA, pH7.5	770ml	59	69.0	45430	0.85	75
(4) DEAE-cellulose column chromatography	1.4 litres	31.4	13.0	43960	2.4	73
(5) Calcium phosphate at pH7.5 used batchwise	1.4 litres	31.0	8.0	43400	3.8	72
(6) $(NH_4)_2SO_4$ taken to 60% saturation. Precipitate spun and dialysed against Tris–HCl–EDTA, pH7.5	183ml	161	37.5	29463	4.3	49
(7) CM-cellulose 11 chromatography at pH6.5. Gradient elution. Fractions with specific activity >10 units/mg were pooled	128ml	162	14.5	20763	11.2	34
(8) $(NH_4)_2SO_4$ taken to 60% saturation. Precipitate spun and dialysed against Tris–HCl–EDTA–mercaptoethanol, pH7.5	42ml	435	34.0	18270	12.8	30
(9) CM-cellulose 52 column chromatography at pH6.5. Gradient elution. Fractions with specific activity >16 units/mg were pooled	7ml	750	44.6	5250	16.8	9

Table 2. *Purification procedure for aldolase from 5kg of human liver*

Stage	Volume	Units/ml	Protein (mg/ml)	Total units	Units/mg of protein	% yield
(1) Extraction in 1mM-Tris–HCl–1mM-EDTA–5mM-mercaptoethanol, pH7.5	12.1 litres	0.36	45	4356	0.008	100
(2) pH adjusted to 5.4, $(NH_4)_2SO_4$ precipitation at pH7.4	2.3 litres	1.7	95	3910	0.018	90
(3) DEAE-cellulose column chromatography. Fractions with specific activity >0.2 unit/mg were pooled. $(NH_4)_2SO_4$ taken to 60% saturation	13 litres	0.2	1.1	2600	0.18	60
(4) Dialysis against Tris–HCl–EDTA–mercaptoethanol, pH7.5. CM-cellulose column chromatography	300ml	5.3	14	1590	0.38	37
(5) Fractions with specific activity >1.0 unit/mg were pooled	240ml	4.7	4.2	1128	1.1	26

weeks). Aldolase from human liver crystallized as needles at room temperature from a 10mg/ml solution of the enzyme in 0.1M-Tris–HCl–2mM-EDTA–2mM-mercaptoethanol, pH7.0, on the addition of saturated $(NH_4)_2SO_4$ to turbidity. The needles, shown in Plate 2(*a*), grew in about 3 weeks. At 4°C in the same buffering system small well-formed prisms were visible after 1–2 months (Plate 2*b*). Large crystals of human muscle aldolase (1.2mm in the longest dimension) were grown for X-ray-diffraction studies. The crystals were examined by using an Elliott rotating anode tube run at 2kW. The 9° precession photographs of the plate-like crystals showed them to be monoclinic, space group $P2_1$ with unit-cell dimensions $a = 16.37$nm, $b = 5.85$nm, $c = 8.16$nm, $\beta = 103.7$°C.

The bipyramid crystals showed hexagonal symmetry and had the space group $P6_222$ with unit-cell dimensions $a = 16.1$nm, $c = 16.9$nm. The monoclinic and hexagonal crystals from the human muscle enzyme were similar in space group, intensity distribution and unit-cell dimensions to the corresponding crystals grown from rabbit muscle aldolase (Eagles *et al.*, 1969).

pH optimum

The pH optimum for the assay was determined over the range pH5.5–9.5 by using 0.1M-Tris–HCl and 50mM-sodium phosphate buffers. With fructose 1,6-diphosphate as substrate the optimum of liver aldolase was pH7.2 and was slightly lower than the optimum for the muscle enzyme, which was between pH7.5 and 7.7. These values did not alter in the buffers studied. No attempt was made to determine whether the fall-off on either side of optimum was due to limitations in the coupling enzymes in the assay or to changes in K_m or V for aldolase.

Kinetics

For the kinetic measurements the activity of liver aldolase was assayed at pH7.2 and muscle aldolase at pH7.5. Triose phosphate isomerase and glycerol 1-phosphate dehydrogenase were dialysed before use. From Lineweaver–Burk plots the K_m for fructose 1,6-diphosphate was found to be 2.7×10^{-5}M and 2.5×10^{-6}M for the muscle and liver enzymes respectively. With fructose 1-phosphate the K_m values were 2.7×10^{-2}M for muscle aldolase and 1.2×10^{-3}M for liver aldolase. For the muscle enzyme the activity ratio of fructose 1,6-diphosphate/fructose 1-phosphate was 38 and for the liver enzyme 1.6. These values together with measurements of the maximum velocity of the reactions are given in Table 3.

pH stability

The stability with respect to pH for aldolase from muscle and liver was studied in 10mM-Tris–HCl and 10mM-sodium phosphate buffers over the range pH4.0–10.0. Samples containing 0.2mg of protein were incubated for 15h at 24°C. They were then assayed under the standard conditions described in the Experimental section. The addition of the sample

Table 3. *K_m and $k_{cat.}$ values for some mammalian aldolases*

B_1 and B_2 denote two types of aldolase from rabbit liver (for details see the text).

Source	K_m for fructose 1,6-diphosphate (M)	$10^{-2}k_{cat.}$ for fructose 1,6-diphosphate (min^{-1})	K_m for fructose 1-phosphate (M)	$10^{-2}k_{cat.}$ for fructose 1-phosphate (min^{-1})	Reference
Rabbit muscle	6.2×10^{-5}		4.3×10^{-3}		Dreschsler *et al.* (1959)
	6.1×10^{-5}	53	1.2×10^{-2}	1.1	Rutter *et al.* (1963)
Chicken muscle	4.2×10^{-5}	38.5	1.7×10^{-2}	1.3	Marquardt (1969)
Rat muscle	1.0×10^{-5}		9.1×10^{-3}		Gracy *et al.* (1970)
	6.2×10^{-5}	57	9.0×10^{-3}	1.3	Ikehara *et al.* (1970)
Human muscle	2.7×10^{-5}	26.5	2.7×10^{-2}	0.7	Present study
Rabbit liver (B_1)	1.2×10^{-6}	2.3	8.7×10^{-4}	4.6	Rutter *et al.* (1963)
Rabbit liver (B_2)	2.3×10^{-6}	4.0	8.3×10^{-4}	4.6	Rutter *et al.* (1963)
Rat liver	4.0×10^{-6}	4.7	2.0×10^{-3}	4.5	Ikehara *et al.* (1970)
	1.6×10^{-7}		1.5×10^{-3}		Gracy *et al.* (1970)
	3.9×10^{-6}		2.4×10^{-3}		Matsushima *et al.* (1968)
Human liver	3.0×10^{-6}		3.0×10^{-3}		Gürtler *et al.* (1971)
	2.5×10^{-6}	1.7	1.2×10^{-3}	1.1	Present study

did not alter the pH of the assay medium. Nearly 100% of the starting activity was retained for both liver and muscle enzymes over the pH range 6.5–9.0, but beyond this range the activity declined sharply.

Incubation of samples containing 0.2–0.5mg of muscle aldolase at 54°C for 15min resulted in a difference in the pH profiles for Tris and sodium phosphate buffers (Fig. 1). Incubation was performed in 20mM buffer in a constant-temperature water bath and the pH of the sample checked during the incubation. After heating, samples were cooled on ice and then a portion was taken for assay. Occasionally the pH of the sample was adjusted before being assayed and the pH of the assay medium was always checked on completion of the assay. Phosphate buffer protects the enzyme over a wide pH range, having its maximum effect at pH6.9, whereas Tris buffer protects over a small range and is maximal at pH8.0. Incubation with 50mM-$(NH_4)_2SO_4$ with the 50mM-Tris–HCl buffer shifted the curve so that it resembled the phosphate buffer curve. Similar stability curves could also be obtained with liver aldolase under the same conditions except that the enzyme was incubated at 48°C.

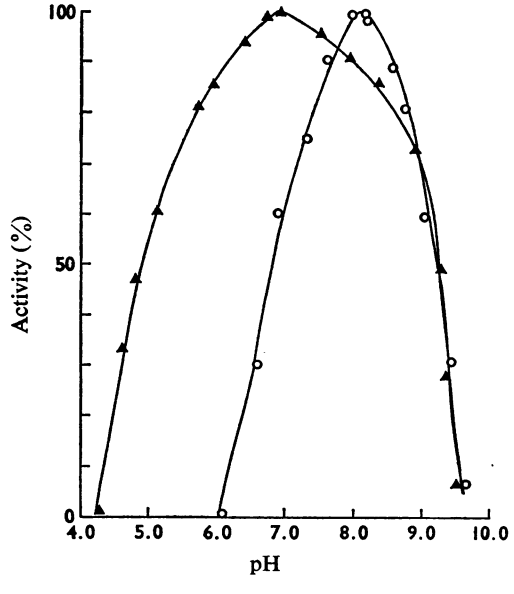


Fig. 1. *Protective action of phosphate on the muscle enzyme*

Enzyme (0.5mg of protein) was incubated at 54°C in 50mM-Tris–HCl buffer (○) and in 50mM-sodium phosphate buffer (▲). For the phosphate curve, results are expressed as a percentage of the activity at pH6.9 and for the Tris curve at pH8.0.

Temperature stability

The temperature profiles for aldolase from human muscle and liver are shown in Fig. 2. Samples (5ml, 0.1mg/ml) of enzyme were incubated in either 50mM-Tris–HCl buffer, pH8.0, or 50mM-sodium phosphate buffer, pH6.5, for 15min. Incubation of a 1mg/ml solution of protein for 1h in 50mM-sodium phosphate, pH7.0, at 53°C resulted in complete inactivation of the liver enzyme and a 70% loss in the activity of the muscle enzyme. In 50mM-Tris–HCl buffer the profiles for both enzymes were essentially the same as those with phosphate buffer although the absolute activities were lower.

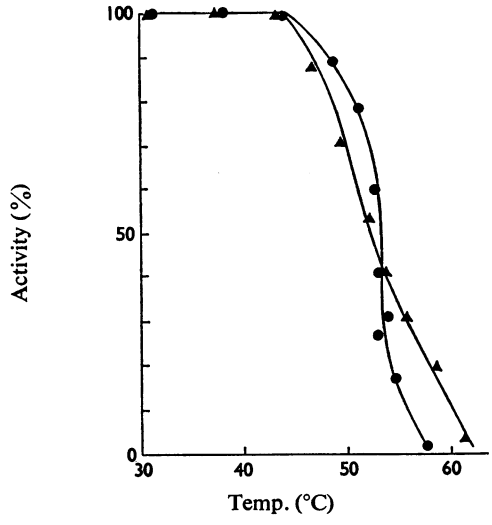


Fig. 2. *Temperature stability of aldolase from human muscle* (▲) *and liver* (●)

Activity is expressed as the percentage of the activity at 25°C. See the text for details.

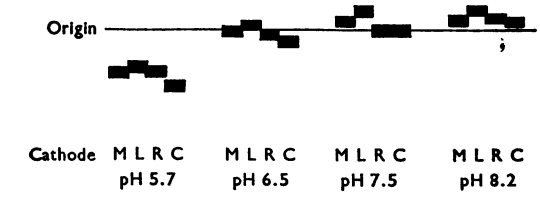


Fig. 3. *Electrophoretic pattern of various mammalian aldolases on cellulose acetate strips*

M, Human muscle aldolase; L, human liver aldolase; R, rabbit muscle aldolase; C, chicken muscle aldolase. The conditions were similar to those given in Table 4.

Electrophoresis

Electrophoresis was performed at four different pH values, 5.7, 6.5, 7.5 and 8.2, in 20mM-sodium phosphate buffer on cellulose acetate paper. The buffers were prepared by mixing 0.1M solutions of sodium monophosphate and diphosphate to the appropriate pH value (10°C) and then diluting. The pattern (Fig. 3) shows that the net charge on the liver enzyme is similar to that of muscle although slightly more negatively charged under the conditions used. Aldolase from rabbit and chicken muscle behaved in a similar manner to the human enzymes. The relative mobilities are given in Table 4.

Tryptic peptide 'mapping'

The tryptic peptide 'maps' of aldolase from human liver and muscle were complex. Muscle aldolase had more neutral peptides than liver and in general the maps from these two enzymes were quite

Table 4. *Relative mobilities of aldolase from human muscle, human liver, rabbit muscle and chicken muscle on cellulose acetate*

Electrophoresis was performed in 20mM-sodium phosphate buffers. The buffer concentration was the same for the strip and the electrode vessels. The cellulose acetate strips (2.5cm × 20cm) were run for 90min at 200V at 10°C with a distance of 13cm between wicks. Mobility is expressed as migration (in cm) from the origin and is positive for migration towards the cathode.

pH	Human muscle	Human liver	Rabbit muscle	Chicken muscle
5.7	1.1	1.0	1.1	1.4
6.5	0.1	−0.1	0.2	0.3
7.5	−0.2	−0.5	0.0	0.0
8.2	−0.3	−0.5	−0.4	−0.2

Table 5. *Comparison of amino acid analyses of human muscle and liver aldolase with rabbit muscle and liver aldolase*

The results are expressed as residues/158000mol.wt.

Amino acid	Human liver*	Human liver†	Rabbit liver‡	Rabbit liver B_1§	Rabbit liver B_2§	Human muscle*	Rabbit muscle‡
Lysine	105.1	106.8	110.8	112	115	100.1	104
Histidine	26.9	35.3	34.0	41	35	34.5	44
Arginine	59.9	57.9	58.5	65	65	50.2	56
Aspartic acid	131.9	133.7	141.9	141	145	114.9	116
Threonine	63.5	77.9	74.9	78	76	84.1	88
Serine	80.2	77.1	59.8	64	67	85.6	84
Glutamic acid	181.5	175.2	165.2	173	163	184.7	164
Proline	56.7	66.8	68.8	61	66	74.5	80
Glycine	116.5	115.1	116.7	117	110	129.8	124
Alanine	137.7	172.4	149.4	144	134	159.1	172
Half cystine	16.4	16.0	30.2	31	20	30.2	32
Valine	102.6	94.7	101.3	94	90	87.1	80
Methionine	22.7	24.4	25.2	20	17	12	12
Isoleucine	80.4	79.9	81.0	76	73	71.6	76
Leucine	152.1	135.5	141.9	135	141	150.2	140
Tyrosine	42.1	36.9	34.9	38	38	50.1	48
Phenylalanine	46.1	35.9	38.1	44	48	32.6	28
Tryptophan	13.2	13.5	16.0	15	12	13.1	12

* The results for threonine and serine are corrected for destruction by extrapolation to zero time; the values for valine, leucine and isoleucine were obtained from the 72h hydrolysis. The SH content was determined by 5,5′-dithiobis-(2-nitrobenzoic acid) titration as described in the Experimental section.

† Values of Gürtler *et al.* (1971) corrected for mol.wt. 158000.

‡ Values of Gracy *et al.* (1969).

§ Values of Rutter *et al.* (1963) for the two types, B_1 and B_2, of rabbit liver aldolase.

different. Some similarities, however, in peptide colour and position were observed in about five of the very basic peptides. A total of 57 peptides could be detected for human muscle aldolase and 45 for the liver enzyme.

Amino acid analysis

The amino acid composition of aldolase from rabbit and human muscle and liver is shown in Table 5, together with the composition found by Gürtler *et al.* (1971) for human liver aldolase.

Discussion

Aldolase was purified from human muscle and liver and various properties of the enzymes were compared. The methods of preparation can be used on large quantities of tissue (20kg or more) and have been used successfully to isolate aldolase from other animal tissues, e.g. rabbit, chicken and horse. This procedure for preparing human aldolase differs from preparations of aldolases from other mammalian muscles mainly because of the low activity of this enzyme in the initial extract; more stages are therefore needed and larger amounts of tissue must be used to obtain a pure enzyme. The poor yield of aldolase obtained by extracting the muscles in low-ionic-strength buffer is not due to binding of the enzyme to the structural proteins of muscle as observed by Arnold & Pette (1968). Over 80% of the total aldolase present in human muscle can be extracted by our method. All of the enzymes prepared by this method were pure as judged by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate.

During the preparation of liver aldolase two peaks were occasionally eluted from the CM-cellulose column: one peak (aldolase I), which contained variable amounts of aldolase activity, was eluted whilst the column was being washed with buffer and the second peak, which contained the bulk of aldolase activity (aldolase II), was eluted only after the application of a salt gradient; these fractions when chromotographed separately also produced similar results. The aldolase I fraction sometimes accounted for approximately one-third of the aldolase applied to the column. The aldolase I fraction was not adsorbed on calcium phosphate gel. In contrast, most of the liver aldolase (aldolase II) was adsorbed on CM-cellulose at pH6.5 and on calcium phosphate gel. The physical and kinetic properties of aldolase I and aldolase II studied were similar.

Isoenzymes have been observed in human liver (Anstall *et al.*, 1966) and two forms of liver aldolase (B_1 and B_2) with similar structural properties and differing kinetic parameters have been isolated from rabbit liver (Rutter *et al.*, 1963). Fractions aldolase I and II may represent two distinct naturally occurring forms of aldolase in human liver; two facts, however, argue against this. First, the proportions of the two aldolases vary from one preparation to another. There is some correlation between the amount of aldolase I and the time for which the tissue had been stored, aldolase I increasing on storage. Secondly, a similar phenomenon of two peaks with aldolase activity eluting from CM-cellulose was encountered during the preparation of aldolase from rabbit muscle that had been stored frozen. It therefore appears that some modification of the protein, e.g. proteolytic digestion by carboxypeptidase (Lacko *et al.*, 1970), occurs during storage or perhaps isolation.

Two crystal forms of aldolase were prepared from both human liver and muscle aldolase. Mammalian muscle aldolases in general will crystallize in two forms: a plate-like form that grows at room temperature and a hexagonal form that only grows in the cold (4°C). For muscle aldolase plates grow best from solutions at pH7–8 and hexagonal bipyramids from those at pH6.5–7.5. Only one crystal form has so far been reported for mammalian liver aldolase; this is needle-shaped and has been grown from ox (Peanasky & Lardy, 1958), rabbit (Gracy *et al.*, 1969), rat (Matsushima *et al.*, 1968) and human liver (Gürtler & Leuthardt, 1969). Human liver aldolase will also crystallize as prisms at 4°C from a variety of buffers.

From examination of the X-ray-diffraction patterns of aldolase from muscle of chicken, rabbit, ox, horse and human it is possible to determine the degree of structural similarity between these various molecules. All the species crystallize in a monoclinic and hexagonal space group ($P2_1$ and $P6_222$ respectively) and show many similarities in intensity distribution and unit-cell dimension, which suggests that these mammalian aldolases are similar in quaternary structure to at least a resolution of 0.5nm (Eagles, 1971).

The pH optimum for the assay of human liver aldolase is pH7.2, which is lower than the value of 7.7 reported by Rutter *et al.* (1963) for rabbit enzyme in a similar assay system. Human muscle aldolase has a pH optimum around 7.5–7.7, a higher value than that reported by Marquardt (1969) for the chicken muscle enzyme (pH7.1).

The K_m values for the human aldolases closely resemble the values reported for other mammalian aldolases. The K_m for human liver aldolase was, however, half the value reported by Gürtler *et al.* (1971) with fructose 1-phosphate as substrate. The $k_{cat.}$ values of both the human liver and muscle enzymes were lower than those for other aldolases. These differences may be real, but they may be caused by modification of the enzymes during storage or isolation of the tissue by carboxypeptidase activity (Lacko *et al.*, 1970). For example Rutter *et al.* (1963) showed that the catalytic activity of aldolase from both rabbit muscle and bovine liver was considerably

decreased by prior treatment with carboxypeptidase.

The greater stability of aldolase in the presence of P_i or of phosphate-containing compounds, e.g. fructose 1,6-diphosphate and pyridoxal phosphate, has been observed by a number of workers. Thus Szanjáni *et al.* (1970) showed that P_i protects against inactivation by bromoacetate and Eagles *et al.* (1969) showed that both phosphate and sulphate bind to aldolase to protect thiol groups from reacting with 5,5′-dithiobis-(2-nitrobenzoic acid). Phosphate and sulphate anions both protect aldolase from human muscle and liver against denaturation by heat, presumably by binding at or near the active site of these enzymes. The stability profiles of both enzymes are very similar and provide little means of distinguishing between these two enzymes. At temperatures greater than 55°C, human muscle aldolase is noticeably more stable than the liver enzyme. The effect of heat can therefore provide an easy means of distinguishing them. By carefully defining the conditions with respect to buffer, ionic strength, pH, temperature and time of incubation it is possible to determine the proportion of either isoenzyme in a mixture of the two by assaying before and after heat treatment.

From the amino acid analysis only small differences in the serine and threonine content were found after 48 and 72h of hydrolysis and the values were extrapolated to zero time. The values for valine, leucine and isoleucine differed appreciably during hydrolysis and a 76h hydrolysis time was arbitrarily chosen to determine the values for these amino acids. The value of 12 residues of methionine/mol of human muscle enzyme was obtained from results of cyanogen bromide treatment of the enzyme. The amino acid analysis consistently gave lower values than this.

Thiol group titrations with 5,5′-dithiobis-(2-nitrobenzoic acid) consistently gave values of around 30.2 SH groups/mol of muscle enzyme. A similar value was also obtained when rabbit muscle aldolase was titrated under similar conditions. The amino acid analysis of the rabbit enzyme (Lai, 1968) indicated the presence of 32 SH groups/mol. A number of workers have shown that low values for SH group determinations on aldolase were obtained with 5,5′-dithiobis-(2-nitrobenzoic acid). Steinman & Richards (1970) suggested that this may be due to the oxidation of some of the SH groups as the molecule unfolds in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid). Despite these precautions, however, low values for SH group determinations may be obtained. With the liver enzyme 16 SH groups were found per molecule.

The amino acid composition of human liver aldolase found in this study is similar to that found by Gürtler *et al.* (1971) although large differences were observed for threonine, alanine and leucine, proline and phenylalanine. In general the composition for the human liver enzyme is somewhat similar to the composition of both aldolase B_1 and B_2 from rabbit liver reported by Rutter *et al.* (1963). There is, however, greater similarity between the composition of the enzyme from human liver and that reported for rabbit liver aldolase by Gracy *et al.* (1969).

In general aldolases from muscle tissues form a closely homologous group of enzymes (Anderson *et al*, 1969) and work on the active-site peptide from rabbit liver aldolase suggests that this homology is not tissue-specific. Although homology between proteins tends to be hidden in peptide 'maps' the results from these studies together with the amino acid analyses show that there are significant differences in the primary structures of aldolase from human liver and muscle. Further work must, however, be done before the extent of homology between the human isoenzymes can be estimated.

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