3 U. MAITRA AND E. E. DEKKER, *J. Biol. Chem.*, 238 (1963) 3660.
4 G. BRANDNER AND A. I. VIRTANEN, *Acta Chem. Scand.*, 18 (1964) 574.
5 R. K. MORTON AND J. R. E. WELLS, *Nature*, 201 (1964) 477.
6 K. KURATOMI AND F. FUKUNAGA, *Biochim. Biophys. Acta*, 78 (1963) 617.
7 U. MAITRA AND E. E. DEKKER, *J. Biol. Chem.*, 239 (1964) 1485.
8 R. G. ROSSO AND E. ADAMS, *Biochem. Biophys. Res. Commun.*, 23 (1966) 842.
9 Y. SEKIZAWA, M. E. MARAGOUDAKIS, T. E. KING AND V. H. CHELDELIN, *Biochemistry*, 7 (1966) 2392.
10 A. RUFFO, A. TESTA, A. ADINOLFI AND G. PELIZZA, *Biochem. J.*, 85 (1962) 588.
11 E. ADAMS AND A. GOLDSTONE, *Biochim. Biophys. Acta*, 77 (1963) 133.
12 T. YONEYA AND E. ADAMS, *J. Biol. Chem.*, 236 (1961) 3272.
13 Y. SEKIZAWA, M. A. MARAGOUDAKIS, S. S. KERWAR, M. FLIKKE, A. BAICH, T. E. KING AND V. H. CHELDELIN, *Biochem. Biophys. Res. Commun.*, 9 (1962) 361.
14 B. PAYES AND G. LATIES, *Biochem. Biophys. Res. Commun.*, 10 (1963) 460.
15 B. PAYES AND G. LATIES, *Biochem. Biophys. Res. Commun.*, 13 (1963) 179.
16 D. C. FISH AND H. J. BLUMENTHAL, *Methods Enzymol.*, 9 (1966) in the press.

Received September 19th, 1966

Biochim. Biophys. Acta, 132 (1967) 200–203

BBA 63233

A staining procedure for demonstration of multiple forms of aldolase

In animal tissues two isoenzymes of aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7 or fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) have been differentiated. Aldolase A catalyses the reaction fructose 1-phosphate$\rightleftharpoons$dihydroxyacetone phosphate + glyceraldehyde at about 1/50th of the rate at which it catalyses the reaction fructose 1,6-diphosphate$\rightleftharpoons$dihydroxyacetone phosphate + glyceraldehyde phosphate. Aldolase B utilizes fructose 1-phosphate (Fru-1-*P*) and fructose 1,6-diphosphate (Fru-1,6-P_2) at about the same rate. Measurement of the ratio of aldolase activities (Fru-1,6-P_2/Fru-1-*P*) in different tissues of many species has shown a variation between 50/1 (muscle) and 1/1 (liver)[1,2].

The simplest method of screening tissue extracts for the presence of multiple molecular forms of any enzyme is electrophoresis, followed by staining. No such method has been available for the detection of aldolase, and after electrophoresis the enzyme could only be identified by elution and subsequent spectrophotometric assay. A method has been developed for the detection and identification of multiple forms of aldolase on starch-gel electrophoresis which utilizes the ready reducibility of alkaline silver nitrate by products of aldolase catalysis.

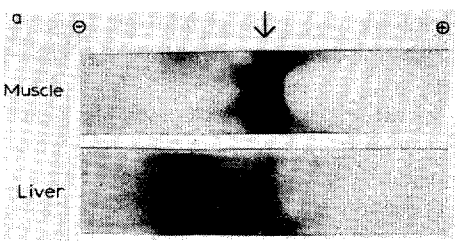
Human tissues were obtained either at operation, or from autopsy when death had occurred within 12 h. Rat tissues were obtained from white Sprague–Dawley or Chester–Beatty hooded rats. Tissues were homogenized at 0° in equal amounts (w/v) of 0.24 M sucrose containing $2 \cdot 10^{-5}$ M EDTA; for muscle, 2 vol. of sucrose solution were used. A crude supernatant fraction was separated after ultracentrifugation at 80 000 × *g* for 30 min.

Electrophoresis was performed on vertical starch gel. For electrophoresis with citrate–phosphate buffer (pH 7.0) the starch gel was made up in a solution of $1.8 \cdot 10^{-3}$ M citric acid and $13 \cdot 10^{-3}$ M Na_2HPO_4, and the "bridge" solution contained $6 \cdot 10^{-3}$ M

citric acid and $48 \cdot 10^{-3}$ M Na_2HPO_4. Undiluted supernatant fraction (0.05 ml, except for muscle where 0.05 ml of a 1:6 dilution in 0.25 M sucrose was used) was applied. A constant current of 2 mA/cm was passed for 18–24 h (voltage 100–150 V). For electrophoresis with Aronsson–Grönwall buffer (pH 8.9) (60.5 g Tris, 6 g EDTA, 4.6 g boric acid per l), the gel was made up using an 1:10 dilution and the bridge solution was an 1:2.5 dilution; electrophoresis was conducted at 1.24 mA/cm constant current (voltage about 200 V). After completion of the run, the gel was sliced, and the strips corresponding to the application slots were separated, either for staining or for extraction.

For staining, each gel strip was overlaid with a strip of Whatman No. 1 filter paper which had been freshly soaked in a 2% (w/v) solution of Fru-1,6-P_2 in 0.1 M acetate–Tris buffer (pH 7.6) containing $2 \cdot 10^{-3}$ M iodoacetate. The gel (containing the aldolases) and the paper (containing the substrate) were incubated together at 37° for a period of 15–60 min, depending on the activity of the enzyme. After incubation, the paper strips were removed and soaked in silver nitrate–acetone solution (0.5 ml saturated aqueous silver nitrate *plus* 100 ml acetone and sufficient water to dispel the precipitate) for 20 sec, dried in air, and transferred to a vessel containing 0.5 M ethanolic NaOH for about 30 sec. Products of aldolase activity appeared as black bands of silver. Background brown colour was removed by soaking the strips in 2% (w/v) aqueous sodium thiosulphate solution, and the strips were finally dried in air.

For extraction of enzyme from unstained gel, the strips of gel were sectioned into segments, usually 0.5 cm or 1 cm long, after the positions of the enzyme bands had been located by staining marker strips. Each fraction was placed in a separate centrifuge tube and crushed in 0.5 ml or 1 ml of 0.1 M ammonium sulphate in 0.1 M ammonium phosphate buffer (pH 7.0), then allowed to stand overnight at 4°. The starch was removed by centrifugation and the supernatant obtained for assay.



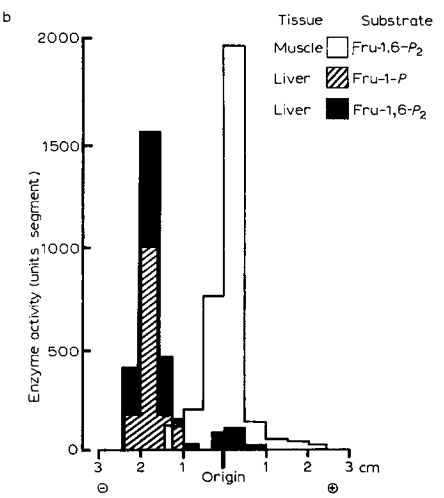


Fig. 1. Comparison of the staining and elution procedures for demonstration of aldolases after starch-gel electrophoresis. (a) A photograph of stained paper strips (using Fru-1,6-P_2 as substrate) which overlaid the starch gel after electrophoresis of liver and muscle extracts is given above. (b) A histogram of enzyme activity (1 unit = 0.001 change in absorbance per min in 0.27 ml final volume), using either Fru-1,6-P_2 or Fru-1-P as substrate, in extracts of corresponding segments of the gel, is given below.

Aldolase activity was assayed, using a Hilger Uvispek with Gilford attachment for automatic recording, by the method modified from RACKER[3], using the aldolase test combination provided by Boehringer which contains $2 \cdot 10^{-3}$ M Fru-1,6-P_2 as substrate. The mixed reagent solution (0.26 ml) was placed in a microcuvette and 0.01 ml of the solution obtained from extraction of the gel was added to start the reaction. Alternatively, in place of Fru-1,6-P_2, $4.5 \cdot 10^{-3}$ M Fru-1-P was used as substrate; however, for estimation of Fru-1-P to Fru-1,6-P_2 ratio in Chester–Beatty hooded rats and for some human tissues the Fru-1-P concentration used was $18.0 \cdot 10^{-3}$ M. Blanks contained all reagents except Fru-1,6-P_2 or Fru-1-P.

Although the action of phosphatases which produce fructose 6-phosphate or fructose from Fru-1,6-P_2 might have been expected to interfere with the staining procedure because of their reduction of the silver nitrate, nevertheless because of the different speeds of the reactions it is possible to distinguish between the rapidly reducing trioses produced by aldolase, and the slowly reducing ketose phosphate and ketose produced by phosphatases. Reduction of silver nitrate by unchanged Fru-1,6-P_2 is very slow.

Fig. 1 shows a comparison of staining and segmental elution procedures for

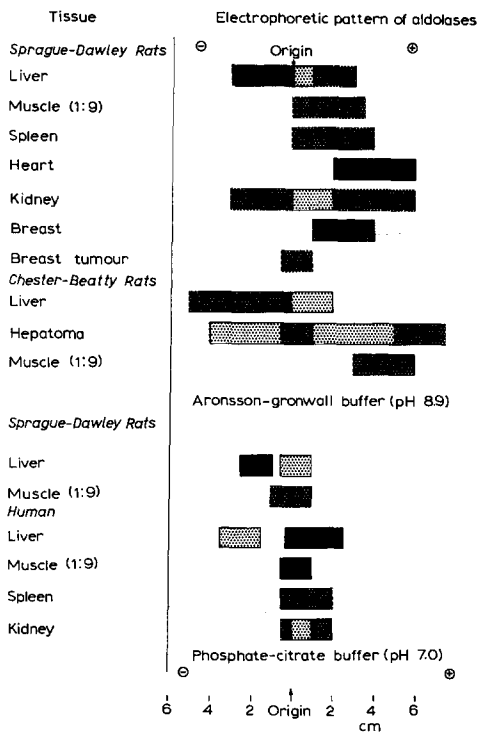


Fig. 2. Distribution of multiple forms of aldolase after starch gel electrophoresis of extracts of rat and human tissues. Sprague–Dawley and Chester–Beatty rat tissues have been examined in Aronsson–Grönwall buffer (above); Sprague–Dawley rat and human tissues have been examined in phosphate–citrate buffer (below). The distribution and relative intensity of the stained bands which represent aldolase activity are shown diagramatically; higher aldolase activities correspond to darker bands. The aldolase patterns are taken from photographs of stained strips, and the distributions have been confirmed by assays of extracted segments of gel.

aldolase after electrophoresis using phosphate–citrate buffer (pH 7.0) of duplicate extracts of liver and muscle from Sprague–Dawley rats. Liver showed one major band towards the cathode and one minor band at the origin, and muscle showed one band at the origin, when Fru-1,6-P_2 was used as the substrate; the positions and intensities of the bands corresponded with the two procedures. When the eluates were assayed using Fru-1-*P* as substrate, only the major liver band reacted and there was negligible aldolase activity in the minor liver band and the muscle band. The major liver band has the substrate activities of aldolase B, and the muscle band those of aldolase A.

Fig. 2 shows the distribution of aldolases in rat and human tissues. Sprague–Dawley rat spleen aldolase resembles that of muscle, as does cardiac muscle; kidney resembles a mixture of muscle and liver. The aldolase of normal post-lactating breast runs towards the anode, whereas that of 9,10-dimethyl-1,2-benzanthracene-induced rat breast tumour remains at the origin. In the same way an extract of transplantable hepatoma in Chester–Beatty rats was compared with normal rat liver and rat muscle: the hepatoma contained a mixture of aldolases, and the principal band ran in the same position as the abnormal aldolase of the breast tumour. Five multiple forms of aldolases have been found in brain[4].

The total aldolase activity of human tissues from recent autopsy was comparable with that of fresh rat tissues so it was assumed that no major loss of aldolase activity had occurred. Separation of the multiple forms could be done at pH 7.0 in phosphate–citrate buffer. Muscle showed one aldolase band at the origin, whereas liver showed a major band at the origin and a minor band towards the anode; kidney had a mixture of two bands. Spleen resembled muscle.

This staining procedure has been found to be entirely satisfactory for the rapid location of aldolase on starch gel and other media.

This work was supported by a grant from the British Empire Cancer Campaign for Research. We are grateful to Dr. C. J. FOXWELL for valuable advice.

Department of Chemical Pathology,
The Royal Free Hospital,
London (Great Britain)

REGINA PIETRUSZKO*
D. N. BARON**

1 W. J. RUTTER, R. E. BLOSTEIN, B. M. WOODFIN AND C. S. WEBER, *Advan. Enzyme Regulation*, 1 (1963) 39.
2 F. SCHAPIRA, *Bull. Soc. Chim. Biol.*, 43 (1962) 1365.
3 E. RACKER, *J. Biol. Chem.*, 167 (1947) 843.
4 C. J. FOXWELL, E. J. CRAN AND D. N. BARON, *Biochem. J.*, 100 (1966) 44P.

Received August 8th, 1966

* Present address: Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A.
** To whom requests for reprints should be made. (The Royal free Hospital, London, W.C. 1).