of plaques was less than 5 percent of the number obtained in tests with B^3/B^3 erythrocytes.
6. A. H. Coons, *J. Cell. Comp. Physiol.* **52**, 55 (1958); G. Attardi, M. Cohn, K. Horibata, E. S. Lennox, *Bacteriol. Rev.* **23**, 213 (1959); ———, *J. Immunol.* **93**, 94 (1964); R. N. Hiramoto and M. Hamlin, *ibid.* **95**, 214 (1965); G. J. V. Nossal and O. Makela, *J. Immunol.* **88**, 604 (1963).
7. L. W. Schierman and A. W. Nordskog, *Nature* **197**, 511 (1963).
8. F. M. Burnet, *The Clonal Selection Theory of Acquired Immunity* (Vanderbilt Univ. Press, Nashville, 1959).
9. J. Lederberg, *Science* **129**, 1649 (1959); R. Owen, in *Genetics Today*, S. J. Geerts, Ed. (Pergamon Press, New York, 1963), pp. 741–46.
10. M. Simonsen, in *Transplantation*, G. E. W. Wolstenholme and M. P. Cameron, Eds. (Little, Brown, Boston, 1962), p. 185.
11. Supported by grants from the Health Research Council of City of New York (U-1612 and U-1673), NSF (GB-3854) and PHS (AM 010036). We thank Mrs. J. Severin, H. Kreissman, and A. Murgo for technical assistance.

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Isozymes of Aldolase

Abstract. *A new method of starch-gel electrophoresis using a technique of staining dependent on enzyme activity has been employed to demonstrate the isozymes of aldolase from a variety of human, rat, and frog tissues. Five of these isozymes were detected in man, seven in the rat, and at least four in the frog. The abilities of these isozymes to cleave fructose-1,6-diphosphate and fructose-1-phosphate were compared.*

Aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphatelyase) catalyzes the cleavage of fructose-1,6-diphosphate (FDP) to triose phosphate. Since there are at least two forms of this enzyme, distinguished by their different abilities to split fructose-1-phosphate (*1–3*), we conducted experiments to determine the existence of aldolase isozymes. By modifying the enzymatic assay originally described by Sibley and Lehninger (*4*), we have developed the first method for demonstrating these isozymes with starch-gel electrophoresis. In principle, the triosephosphates formed by the action of aldolase upon FDP or F-1-P (fructose-1-phosphate) are trapped by hydrazine and detected by the colored product resulting from their reaction with 2,4-dinitrophenylhydrazine.

Tissue samples were obtained from Wistar rats and frogs (*Rana pipiens*), and from human cadavers no later than 12 hours post-mortem. Because the aldolase content varies from one tissue to another, we diluted the tissue homogenates accordingly; the dilutions ranged from 4 g (wet weight) of testis in 4 ml of disodium ethylenediaminetetraacetate (EDTA) to 0.2 g of skeletal muscle in 6 ml of EDTA. By using this technique, we obtained colored spots of similar intensities. Samples were homogenized in a modified Potter-Elvehjem homogenizer with cold 0.1*M* Na_2 EDTA, *p*H 7.5. The homogenates were centrifuged for 1 hour at 20,000*g* and stored at 4°C until they were introduced into the gel. A horizontal gel was prepared with Connaught hydrolyzed starch (106 percent of the recommend amount) and EBT buffer [0.02*M* EDTA, 0.5*M* boric acid, 0.9*M* tris(hydroxymethyl)aminomethane (tris)] at *p*H 8.6 (*5*). Electrophoresis was performed for 20 to 22 hours at 4°C at 3.0 to 4.0 volt/cm.

Reagents used in the staining procedure were: the substrates, 0.1*M* FDP or 0.1*M* F-1-P (sodium salts prepared from the barium salts obtained from Calbiochem) at *p*H 8.6; 0.56*M* hydrazine sulfate, *p*H 8.6; and tris-HCl buffer, *p*H 8.6. The sliced gels were incubated in a mixture of 15 ml of substrate, 5 ml of hydrazine, 50 ml of buffer, and 0.5 g of albumin for 45 minutes at 37°C. Incubation in a slowly shaking water bath reduced incubation time to 35 minutes. After incubation, the substrate was removed by aspiration. The gels were flooded with 2,4-dinitrophenylhydrazine in 0.5*M* HCl and were incubated again for 45 minutes at 37°C. The aldolase spots appeared after treatment of the gel surface with 0.6*N* NaOH for approximately 10 minutes. These spots were red on a yellow background; hence, photographing them was somewhat difficult. The red color was transitory, and the NaOH rapidly hydrolyzed the gel surfaces; preservation was enhanced by the removal of the NaOH

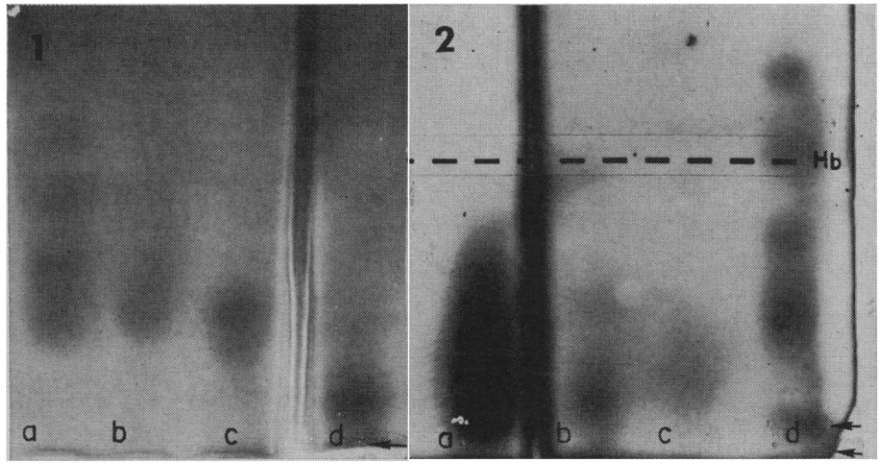

Fig. 1. Isozymes from human cadavers. *a*, Brain; *b*, heart; *c*, rabbit muscle aldolase, control (Boehringer and Soehne, concentration about .008 International Enzyme Units); *d*, human liver. Electrophoresis for 22 hours at 3 volt/cm. The arrow indicates the origin. Fig. 2. Patterns of aldolase from human tissue. *a*, Muscle; *b*, liver; *c*, kidney; *d*, brain. Five isozymes are distinguishable. Mark nearest origin of *d* is an artifact caused by destruction of the gel by NaOH. Hemoglobin (*Hb*) shows as a faint bar in muscle, liver, and kidney tracks. Electrophoresis was carried out for 22 hours at 4 volt/cm. The top arrow points to the artifact; the bottom, to the origin.

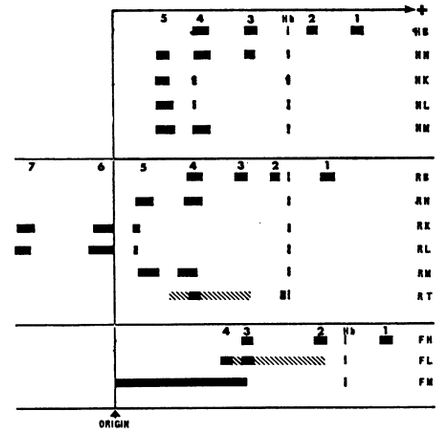

Fig. 3. Scale representation of relative mobilities of aldolase isozymes. Top to bottom: human brain, heart, kidney, liver, and muscle; rat brain, heart, kidney, liver, muscle, and testis; and frog heart, liver, and muscle. Various lengths of bands are probably indicative of quantity of each isozyme. Hb represents hemoglobin bands. (Direction of electrophoretic migration is from origin to right.)

and its substitution by tris-citrate buffer (*p*H 8.5) immediately after the color developed.

When stained, controls lacking substrate (FDP or F-1-P) developed no color. The spot produced by purified aldolase from rabbit muscle corresponded with that located by the amido-black protein stain.

The aldolase isozymes seen thus far are shown in Figs. 1, 2, and 3. We have numbered the spots from anode to cathode. Five isozymes were detected in man, seven in the rat, and at least four in the frog. The aldolase of frog skeletal muscle shows an extensive unresolved band which may consist of more than one component. The rat is peculiar in that its tissues apparently contain seven isozymes, two of which, when subjected to electrophoresis on starch gel, migrate toward the cathode. The species variation can clearly be seen in Fig. 3. While, for a given species, all isozymes may be present in each tissue, their proportions vary, each organ having a typical, reproducible pattern.

It is conceivable that other faint bands of activity were not detected because the total aldolase activity of the different homogenates varied. However, the homogenates of each tissue (at several concentrations, as determined by protein content) have been examined by electrophoresis, and we have found no more than five isozymes in any animal tissue except rat tissue. If the method can be made more sensitive, other forms may possibly be found.

One of the methods used to distinguish between the two previously described forms of aldolase is based on their different affinites for substrates. The "liver aldolase" described by Blostein and Rutter (*2*) has a much higher affinity for F-1-P than does the "muscle aldolase." When F-1-P is substituted for FDP as substrate, only isozymes L-5 and K-5 (Fig. 3) can be detected in human tissue homogenates. In rat tissue homogenates, similar F-1-P substitution shows L-6 and 7 and K-6 and 7 only. Hence, we feel that these isozymes represent the "liver aldolase" described previously. It appears that the patterns of these isozymes in liver and kidney are closely similar.

Previous evidence suggests that aldolase comprises three polypeptide subunits (*3, 6*). More recently, data from detailed studies with the ultracentrifuge have strongly suggested the occurrence of four subunits (*8*), and results of biochemical investigations lend additional support to this view (*9*). The occurrence of five isozymes of aldolase could be more easily explained if there were four polypeptide subunits rather than three; by analogy, one may cite the example of lactate dehydrogenase in this context. The two negatively migrating isozymes detected in the rat are difficult to explain. They are, however, consistently found, and they exhibit activity with either FDP or F-1-P as their substrate.

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References and Notes

1. R. T. Peanasky and H. A. Lardy, *J. Biol. Chem.* **233**, 365 (1958); E. R. Drechsler, P. D. Boyer, A. G. Kowalsky, *ibid.* **234**, 2627 (1959); W. J. Rutter, in *The Enzymes*, P. D. Boyer, H. A. Lardy, K. Meyerback, Eds. (Academic Press, New York, 1961), vol. 5, p. 341.
2. R. E. Blostein and W. J. Rutter, *J. Biol. Chem.* **238**, 3280 (1963).
3. W. J. Rutter, D. M. Woodfin, R. Blostein, *Acta Chem. Scand.* **17** Suppl., 226 (1963).
4. J. A. Sibley and A. L. Lehninger, *J. Biol. Chem.* **177**, 859 (1949).
5. S. H. Boyer, D. C. Fainer, M. A. Naughton, *Science* **140**, 1228 (1963).
6. W. J. Rutter, R. E. Blostein, B. M. Woodfin, C. S. Weber, in *Advances in Enzyme Regulation*, G. Weber, Ed. (Pergamon Press, New York, 1963), vol. 1, pp. 39–56.
7. W. C. Deal, Jr., W. J. Rutter, K. E. Van-Holde, *Biochemistry* **2**, 246 (1963).
8. K. Kawahara and C. Tanford, *ibid.* **5**, 1578 (1966).
9. T. V. Rajkumar, E. Penhoet, W. J. Rutter, *Fed. Proc. Abstr.* **1966**, 1878 (1966).
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Allomorphic Forms of Bacteriophage φX-174 Replicative DNA

Abstract. *The bacteriophage φX-174 replicative form of DNA exists in two configurations, as judged by electron microscopy and sucrose density gradient sedimentation, and on methylated albumin–coated kieselguhr column chromatography. The predominant 21S form of the DNA is a tightly twisted circle. Treatment with deoxyribonuclease causes a single scission in one of the two strands, resulting in an open circle which sediments at 16S.*

Two classes of DNA molecules are observed when the bacteriophage φX-174 replicative double-stranded form of DNA (RF-DNA) is isolated from its bacterial host (*1*). When this RF-DNA was purified by sucrose density-gradient sedimentation, it separated into two components. Approximately 90 to 95 percent of these molecules sedimented with an s_{20} value of 21; the remainder, with 16. These two forms could also be separated by a column of methylated albumin–coated kieselguhr (Fig. 1). Both appeared to be almost equally infective on *Escherichia coli* spheroplasts, and they had an identical buoyant density in neutral CsCl. These data agree with those reported by Jansz and Pouwels (*2*).

Similar results were obtained with SV40 viral DNA (*3*) and with polyoma DNA (*4*). With the polyoma virus, Vinograd *et al.* (*4*) demonstrated that the faster-sedimenting 20*S* polyoma DNA molecules have a highly twisted circular form. They and others (*2*) demonstrated that, upon exposure to very dilute pancreatic deoxyribonuclease, one of the two strands was cleaved and the molecules sedimented at 16*S*. Examination of the 16*S* fraction in the electron microscope showed that the molecules were open circles. On the basis of the polyoma data, we thought it likely that the two differently sedimenting forms of φX-174 RF-DNA also might be due to a similar shift in conformation. As shown below, this is the case.

When purified 21*S* RF-DNA was treated with very dilute pancreatic deoxyribonuclease, the concentration of the 21*S* form decreased with a con-

Table 1. Deoxyribonuclease conversion of 21*S* to 16*S* DNA. Purified 21*S* RF-DNA was treated with pancreatic deoxyribonuclease (reaction mixture as in Fig. 2). At the intervals noted, samples were analyzed by electron microscopy (Fig. 3) and sucrose-gradient sedimentation (Fig. 2).

Treatment time (min)	No. of molecules counted in electron micrographs			21*S* by sedimentation (%)
	Open form	Closed form	Percentage closed	
0	307	3370	91.7	95
2	343	2190	86.5	78
5	1312	999	43.2	50
10	3310	710	17.6	26
15	2855	287	9.1	13
20	3053	149	4.6	6