# Distribution of Fructose Diphosphate Aldolase Variants in Biological Systems*

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ABSTRACT: (1) Three parental fructose diphosphate aldolases, A, B, and C, were detected in tissues of each of 13 vertebrate species tested (including man). The following criteria of identification were employed: relative electrophoretic mobilities to the well-studied rabbit and rat prototype enzymes; substrate specificities (fructose diphosphate/fructose 1-phosphate activity ratio); tissue distributions, and, in some cases, specific immunological properties. A fourth parental aldolase form was detected in trout and salmon but not in other fish studied. (2) In vertebrate tissues containing two of the parental aldolases, five-membered hybrid sets were usually detected, indicating that both aldolases were formed in individual cells. Five-membered hybrid sets were detected in some tissues of all species examined. This suggests that the parental aldolases in vertebrates are tetrameric molecules. (3) Aldolases A, B, and C have a segregated tissue distribution. Aldolase A was present in most tissues investigated; aldolase B was detected in liver and kidney; aldolase C was found in brain and somewhat more variably in heart, spleen, and other tissues. (4) Transitions from a predominance of aldolase A to B in liver and kidney and A to C in brain have been observed in man. C to A transitions were observed in chicken muscle and heart, and in human heart. These transitions emphasize the independent regulation of synthesis of the three aldolases. (5) No evidence of multiple tissue-specific aldolases corresponding to those of the vertebrates was found in invertebrates or plants, although aldolase multiplicity was detected in some species. It is proposed, therefore, that the homologous aldolase genes B and C were functionally consolidated in the vertebrates. (6) Single bands of class II aldolase activity were found in all bacteria and fungi tested. In confirmation of previous work, a single class I and a single class II activity was detected in *Euglena*. (7) The aldolase from *Micrococcus aerogenes* was not inhibited by chelating agents, suggesting this enzyme may be a class I aldolase. No class I aldolases have been found previously in microbial systems.

Two distinct types of fructose diphosphate aldolases have been detected in biological systems. Class I and class II aldolases, as they have been termed (Rutter, 1964), are strikingly different in both molecular and catalytic properties. Class I aldolases have been found in animals, plants, protozoans, and algae, while the class II enzymes have been detected in bacteria, fungi, and blue-green algae (Rutter, 1964; Groves, 1962; Rutter and Groves, 1964). Both class I and class II aldolases are found in *Euglena* (Groves, 1962; Rutter, 1964) and *Chlamydomonas* (Russel and Gibbs, 1967).

Multiple forms of class I aldolases have been detected in certain vertebrate tissues (Penhoet *et al.*, 1966; Foxwell *et al.*, 1966; Anstall *et al.*, 1966; Rensing *et al.*, 1967; Herskovitz *et al.*, 1967). Three parental aldolases have been isolated from the rabbit: aldolase A, the classical muscle enzyme (Taylor *et al.*, 1948); aldolase B, from liver (Rajkumar *et al.*, 1967); and aldolase C, recently isolated from brain (Penhoet *et al.*, 1966; E. Penhoet, M. Kochman, and W. J. Rutter, in preparation). These parental aldolases have many structural similarities and appear to be homologous proteins (Rutter *et al.*, 1963b, 1968; Penhoet *et al.*, 1967). Five-membered A–B and A–C hybrid sets have been detected in some mammalian tissues (Rutter *et al.*, 1968; Penhoet *et al.*, 1966). In addition, five-membered hybrid sets can be produced by the binary combination of the parental subunits (Penhoet *et al.*, 1967). These observations suggested that the class I aldolase molecule is tetrameric, a supposition which has been confirmed (Penhoet *et al.*, 1967; Kawahara and Tanford, 1966; Sia and Horecker, 1968; Castellino and Barker, 1968; L. Hass and H. Sine, personal communication).

The specific aims of this study were (1) to determine the general distribution of class I and class II aldolase variants in biological systems (a question relevant to the evolutionary origin of the variants); (2) to enumerate the members of the class I hybrid sets in various systems (a question relevant to the subunit structure of the aldolase molecule); and (3) to define the tissue specificity of the class I variants in diverse animal groups (a question relevant to the physiological function of the class I variants).

## Materials

The biological materials used for this study and their sources are indicated as follows.

*Vertebrate Tissues.* Adult human, at autopsy: Dr. N.

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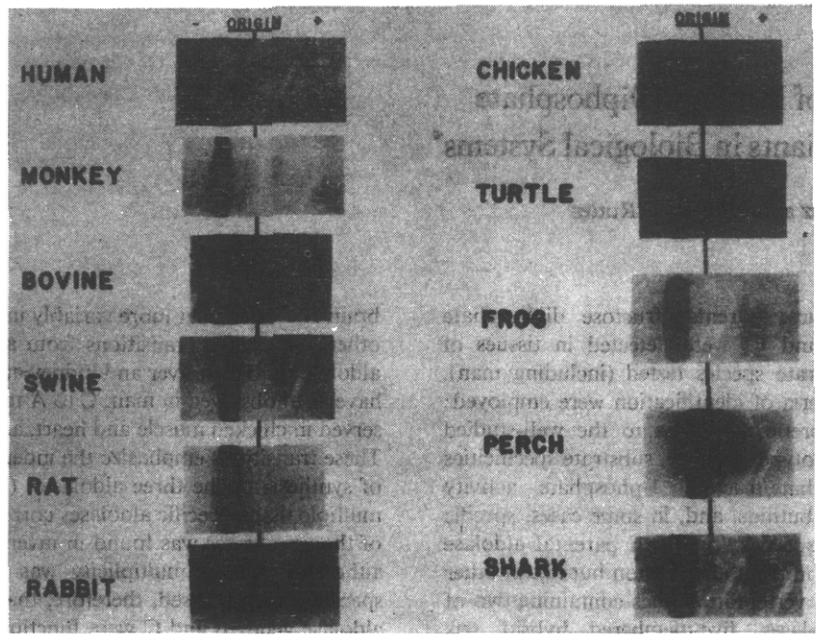


FIGURE 1: Zone electrophoretic resolution of aldolase activities in extracts of skeletal muscle from various vertebrate species. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

K. Mottet, Department of Pathology, University of Washington; embryonic human: Dr. T. H. Shepard, Department of Pediatrics, University of Washington; Rhesus monkey (*Macaca mulatta*): Primate Center, University of Washington; bovine, swine, rat, rabbit and chicken: Lab Associates, Inc., Kirkland, Wash; embryonic chicken: Dr. S. D. Hauschka, Department of Biochemistry, University of Washington; turtles (*Chrysemys picta*) and frogs (*Rana pipiens*): Biological Supply Company, Seattle, Washington; perch (*Embiotoca lateralia*): Seattle Public Aquarium; trout (*Salmo gairdneri*) and salmon (*Oncorhynchus nerka*): Dr. G. Klontz, Western Fish Disease Lab, Seattle, Wash.; and shark (*Squalus sucklei*): Mr. W. Brown, of this laboratory.

*Invertebrate Tissues.* Octopus (*Dofleini martini*): Mr. B. Frick, Sequim, Wash; lobster (*Homarus americanus*): QFC Stores, Seattle, Wash; crayfish (*Pacifastacus trobridgii*): College Biological Co., Seattle, Wash; crab (*cancer magister*): Pure Food and Fish Co., Seattle, Washington; and honey bees (*Apis mellifera*): C. G. Wenner and Sons, Glenn, Calif.

*Plants.* Spinach (*Spinacia oleracea*): QFC Stores, Seattle, Wash; pea (*Pisum sativum*), corn seeds (*Zea maize*), and *Cladophora* sp.: Department of Botany, University of Washington.

*Protists. Protozoa. Tetrahymena pyriformis:* Dr. G. A. Thompson, Department of Biochemistry, University of Washington; and *Euglena gracilis:* cultured by the method of Groves (1962) and Star (1960).

*Fungi.* Yeast (*Saccharomyces cerevisiae*): Standard Brands, Seattle, Washington; and *Neurospora crassa:* Dr. D. R. Stadler, Department of Genetics, University of Washington.

*Bacterial. Bacillus subtilis, Desulfovibrio desulfuricans, Escherichia coli, Pseudomonas multivorans,* and *Micrococcus aerogenes:* Dr. H. R. Whiteley, Department of Microbiology, University of Washington.

## Methods

*Preparation of Extracts.* Extracts of the various biological specimens were prepared by one of the following methods: (1) homogenization in a Potter-Elvehjem homogenizer, (2) freezing and thawing, (3) grinding, (4) sonication, and (5) colloid mill disruption. The procedure giving maximal recovery of activity for each material was used. All extracts were centrifuged at 100,000*g* for 45 min, and the supernatants used for assay.

*Animals.* Most animal tissues were homogenized in 0.05 M Veronal buffer (pH 8.6) (approximately 1 g of tissue/ml of buffer) containing 0.07% $\beta$-mercaptoethanol. Extracts of early embryonic chicken tissues were prepared by freezing and thawing the tissues three times in 0.05 M Veronal buffer (1 g of tissue/ml of buffer).

*Plants.* Pea and corn seeds were washed in 5.25% sodium hypochlorite for 30 min followed by soaking in tap water for 12 hr. The seeds were then allowed to germinate on moist filter paper in the dark. Seedlings (5- to 7-day old) were homogenized in 0.05 M Veronal buffer (2 g of seedlings/ml of buffer). Chopped spinach leaves were ground with a mortar and pestle in the presence of sand and a small amount of 0.05 M Veronal buffer (10 g of leaves/ml). An aliquot of this brei was sonicated for four periods of 30 sec each with a Branson sonifier (9-A setting). Samples of *Cladophora* were prepared as described for spinach without sonication.

*Protists. Protozoa Tetrahymena* were suspended in Veronal buffer (5 g of cells/ml) and the suspension was homogenized. *Euglena* were suspended in 0.1 M Tris (pH 7.5) containing 0.1% $\beta$-mercaptoethanol (approxi-

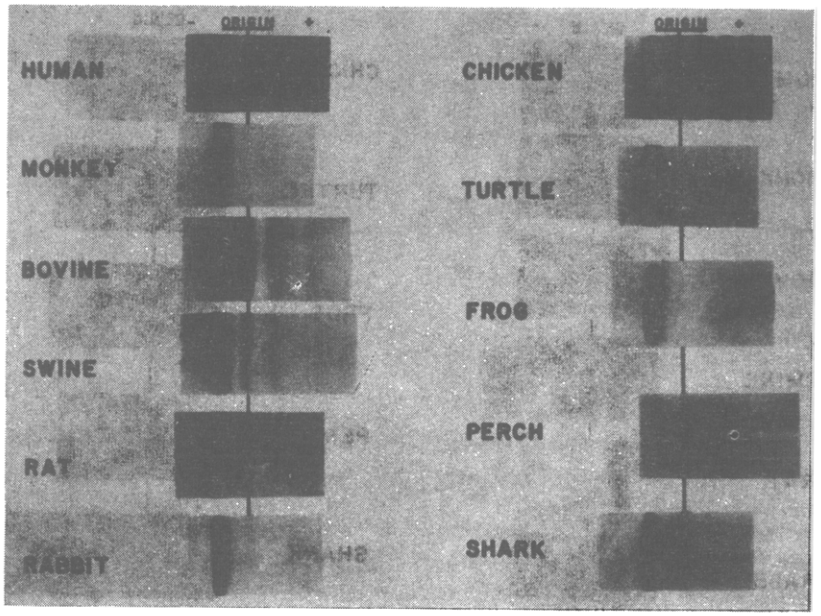


FIGURE 2: Zone electrophoretic resolution of aldolase activities in extracts of spleens from various vertebrate species. The perch profile is faint, but activities corresponding to the A–C set were apparent. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

mately 2 g of cells/ml of buffer) and sonicated as described for spinach.

*Fungi. Neurospora* specimens were homogenized in 0.05 M Veronal buffer (2 g/ml of buffer). Yeast cells were suspended in distilled water (1 g of cells/ml) and ground with glass beads (600 ml of 120-$\mu$ beads plus 1000 ml of suspension) for 20 min in a Gifford-Wood colloid mill.

*Bacteria.* Bacterial extracts were prepared in distilled water by passage through a french pressure cell. The protein concentration of these extracts after centrifugation was approximately 30 mg/ml.

*Activity Measurements.* Aldolase activity was determined by the method of Blostein and Rutter (1963) using either 2.5 mM FDP or 10 mM F-1-P as substrate. A unit of activity is expressed as the cleavage of 1 $\mu$mole of substrate/min per ml of extract (Blostein and Rutter, 1963).

*Electrophoretic Assay.* Cellulose-polyacetate electrophoresis and staining for aldolase activity were performed as previously described (Penhoet *et al.*, 1966). Samples (2–4 $\mu$l) containing 0.002–0.02 unit of aldolase activity were applied to the strips and electrophoresis was performed at 250 V for 90 min. Some extracts, especially those of liver and kidney tissues, produced activity bands on assay plates which contained no FDP. These bands are probably due to alcohol dehydrogenase activity (Shaw and Koen, 1967). In most instances, substitution of the assay buffer with 0.01 M sodium pyrophosphate, 0.005 M $Na_2HAsO_4$, 0.001 M EDTA, and 0.01 M isobutyramide (pH 7.5) prevented the detection of these FDP-independent activities. In addition, the requirement for substrate was checked, and found to be positive for all the aldolase activity bands.

*Immunological Assay.* Chicken antisera against purified rabbit aldolases A and B were prepared by E. Penhoet of this laboratory, using a method similar to that employed by Weber (1965); 1 ml of anti-A sera completely inhibited 120 $\mu$g of pure rabbit aldolase A and 1 ml of anti-B sera completely inhibited 60 $\mu$g of pure rabbit aldolase B.

Twenty miscroliters of human muscle extract containing 0.16 unit of activity was added to 100 $\mu$l of anti-A, anti-B, or distilled water; 10 $\mu$l of human liver extract containing 0.05 unit was added to 200$\mu$l of anti-A, anti-B, or distilled water. All preparations were then incubated for 1 hr at 4°. Following incubation, the preparations were assayed for aldolase activity. Inhibition by antisera was expressed relative to the activity of extracts incubated with water.

## Results

*Identification of Aldolases in Tissues of Various Vertebrate Species.* The identification of the various aldolase activities present in extracts of tissues from the vertebrate species studied is based on several criteria: substrate specificities (FDP/F-1-P activity ratios), electrophoretic mobilities, tissue distributions, and in some instances, specific immunological properties.

The substrate specificity is particularly useful in distinguishing aldolase B from aldolases A and C. The FDP/F-1-P activity ratios of rabbit aldolases A, B, and C are approximately 50, 1, and 12, respectively (Rutter *et al.*, 1968). The activity ratios of the hybrids may be predicted from arithmetic summation of the parental subunits (Penhoet and Rutter, unpublished). Since the low ratio for aldolase B is presumably a reflection of this enzyme's function in the metabolism of fructose (Rutter *et al.*, 1963a), the FDP/F-1-P activity ratio is a suitable diagnostic parameter. The activity ratios of aldolases A and C, on the other hand, are less different, and both are somewhat variable in crude extracts; thus,

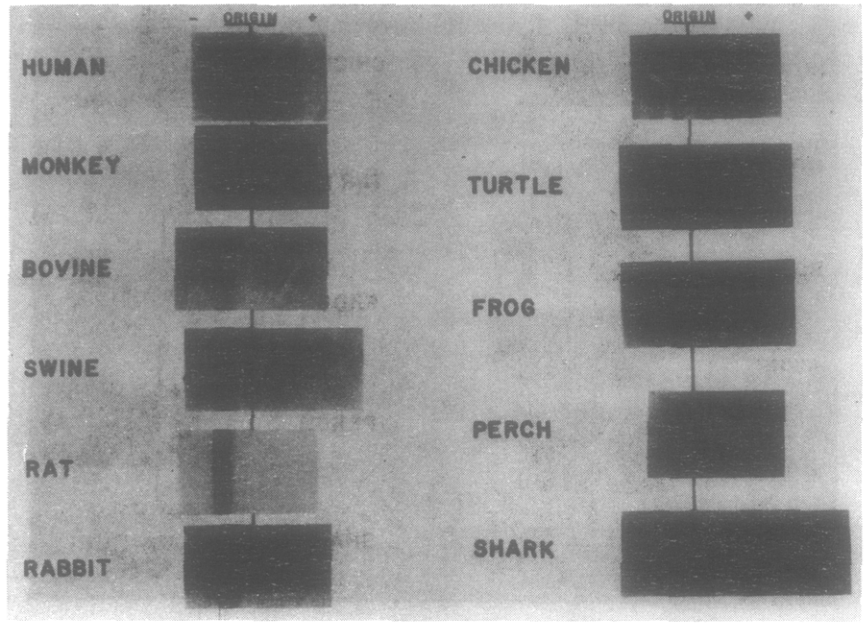


FIGURE 3: Zone electrophoretic resolution of aldolase activities in extracts of hearts from various vertebrate species. The two cathodic activities in shark heart are faint, but the entire A–C set was detected. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

this ratio is of little value in differentiating aldolase A and C in the present studies.

The FDP/F-1-P ratios of skeletal muscle, liver, and kidney of several vertebrates are compared in Table I. The ratios of all muscle activities are high (44–60), characteristic of aldolase A. All of the liver activity ratios are low (1.2–3.5), suggesting high concentrations of aldolase B in these tissues. The intermediate kidney values (4–7) suggest that both aldolases A and B are present in these tissues. The activity ratios of all other tissues tested were considerably higher than those of liver and kidney and suggest that the distribution of aldolase B is restricted primarily to these two tissues.

We also employed the relationship of electrophoretic mobilities of activities detected in tissue extracts to those of the well-studied prototype aldolases A, B, and C isolated from rabbit muscle, liver, and brain, respectively. (Under the conditions employed, rabbit aldolases B, A, and C migrated −1.7, −0.6, and +2.8 cm in 1.5 hr, respectively.) Although this parameter in itself might be a tenuous basis for assignment, in practice it has proved quite useful because of similar electrophoretic relationships between the rabbit parental aldolases and the activities of the other systems tested. In addition, the tissue distribution of the various forms has reinforced the other criteria. Finally, the specific inhibitory properties of antisera directed against the pure rabbit aldolases A and B have been employed to identify these enzymes in human tissues. Even though the parental species do not cross-react to a measurable degree, homologs in the A and B series from other mammals do cross-react with the appropriate antisera prepared against the rabbit enzymes (Weber, 1965).

TABLE I: FDP/F-1-P Ratios of Muscle, Liver, and Kidney from Selected Vertebrates.[a]

| Tissue | Human | Monkey | Rat | Perch | Shark |
|---|---|---|---|---|---|
| Muscle | 44 | 46 | 40 | 60 | 50 |
| Liver | 1.2 | 1.1 | 1.2 | 2.9 | 3.5 |
| Kidney | 4.0 | 4.0 | 3.0 | 5.6 | 7.0 |

[a] Conditions for tissue homogenization and determination of aldolase activity using FDP and F-1-P as substrate are described in the Methods section.

*Electrophoretic Profiles of Aldolase Activity in Vertebrate* Tissues. The activity profiles of aldolase activity in selected tissues from 11 vertebrate sources are presented in Figures 1–7. The trout and salmon profiles were qualitatively different from those of the other vertebrates and are presented separately in Figure 8.

Muscle. With the exception of bovine and swine, a single aldolase activity corresponding to aldolase A was found in all vertebrate skeletal muscle (Figure 1). Two activities with similar mobilities were detected in bovine and swine muscle. For several species, the profiles of different skeletal muscles were compared and found to be identical. All muscle activities tested exhibited a high FDP/F-1-P ratio.

Spleen. Aldolase A was the predominant activity in most spleen extracts (Figure 2); however, several of the A–C hybrids were found in bovine, swine, and perch spleen. The entire A–C set was present in chicken spleen.

Heart. Aldolase A was the only activity detected in bovine, rat, and rabbit hearts. Some members of the A–C set were found in human, monkey, swine, turtle, and perch hearts, while the entire A–C set was present in chicken, frog, and shark hearts.

Brain. As shown in Figure 4, the A–C hybrid set was

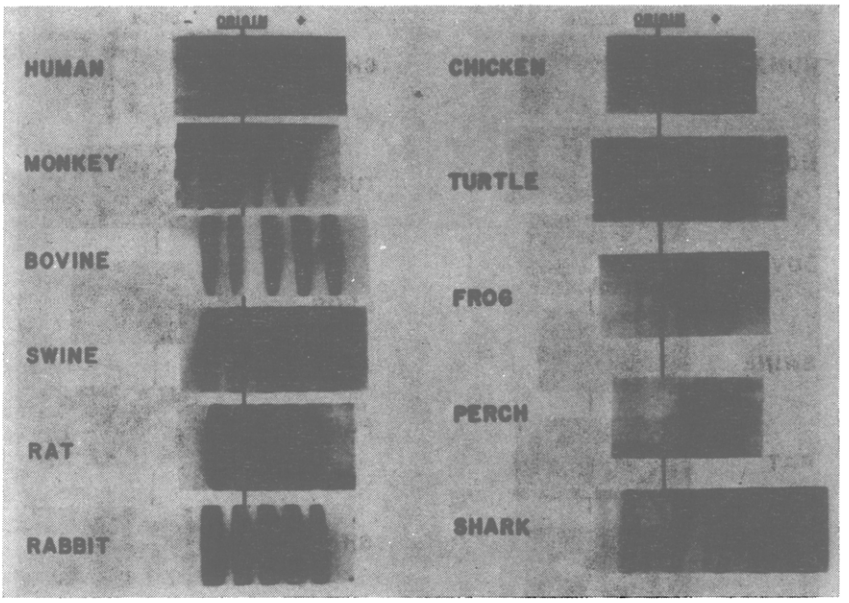


FIGURE 4: Zone electrophoretic resolution of aldolase activities in extracts of brains from various vertebrate species. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

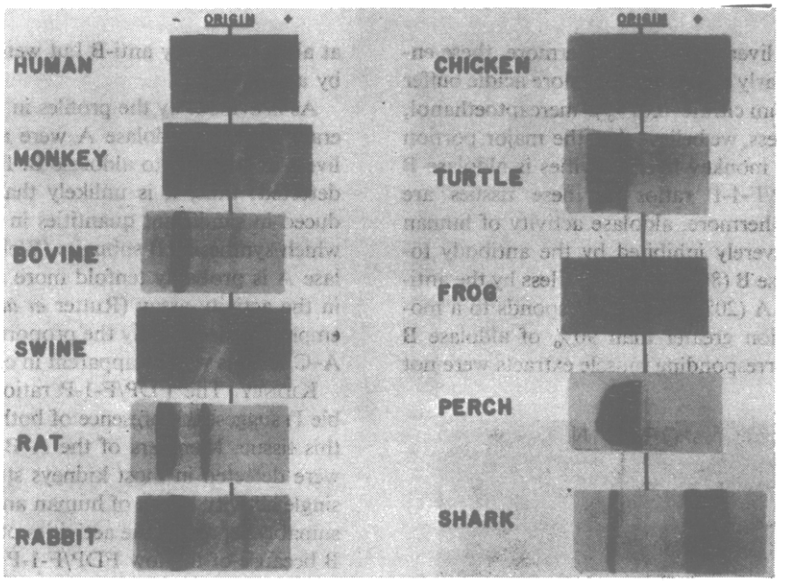


FIGURE 5: Zone electrophoretic resolution of aldolase activities in extracts of livers from various vertebrate species. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

found in all brains tested. The chicken and perch brain profiles were skewed considerably toward the C terminus reflecting a relatively larger concentration of aldolase C subunits in these tissues. No differences between the activity profiles of human brain white and gray matter was detected. In a limited study carried out in collaboration with Dr. H. Kihara, Department of Mental Hygiene, Pacific State Hospital, Pomona, Calif., no qualitative differences were found in the A–C profiles of brain obtained from human beings with a variety of brain disorders, including: Hurler's syndrome, demyelinating encephalopathy, mongolism, microcephalism, hydrocephalism, Tay-Sachs disease, and epilepsy.

LIVER. The liver activity profiles are presented in Figure 5. In most species, aldolase B migrates more cathodically than A. In others (frog and shark) aldolase B appears to be more anodic than A. The anodic band in frog and at least one of the anodic activities in shark are presumably aldolase B since the FDP/F-1-P ratios of frog (Blostein and Rutter, 1963) and shark (Table I) liver extracts are low and because these anodic activities can be easily resolved from the corresponding A–C hybrid sets by coelectrophoresis.

In contrast to the other vertebrates, aldolases A and B of man and monkey have very similar mobilities. These aldolases are poorly resolved by coelectrophoresis

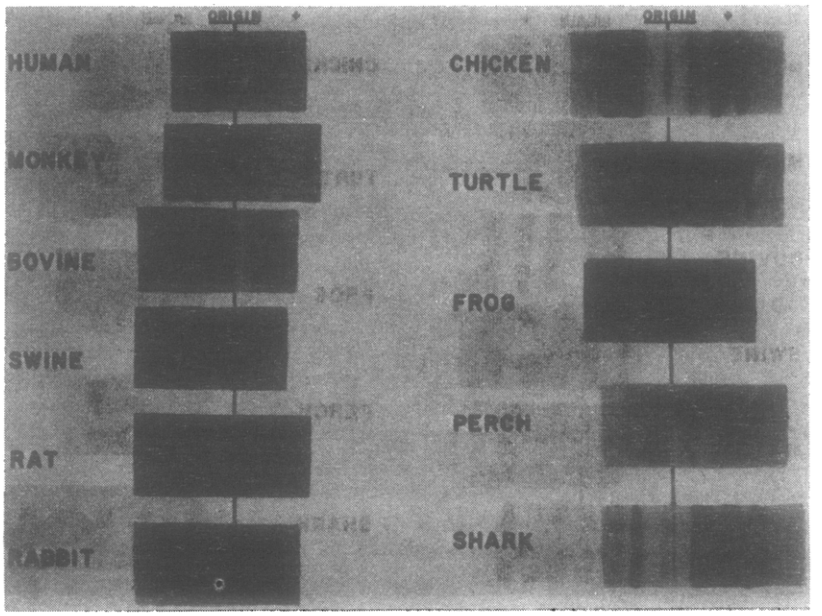


FIGURE 6: Zone electrophoretic resolution of aldolase activities in extracts of kidneys from various vertebrate species. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

of the muscle and liver extracts; furthermore, these enzymes were not clearly separated by a more acidic buffer system (0.1 M sodium citrate–0.07% $\beta$-mercaptoethanol, pH 6.5). Nevertheless, we believe that the major portion of the human and monkey liver activities is aldolase B because the FDP/F-1-P ratios of these tissues are low (Table I); furthermore, aldolase activity of human liver extracts is severely inhibited by the antibody toward rabbit aldolase B (80%) and much less by the antibodies to aldolase A (20%). This corresponds to a molecular concentration greater than 90% of aldolase B in rat liver. The corresponding muscle extracts were not at all inhibited by anti-B but were completely inhibited by antirabbit A.

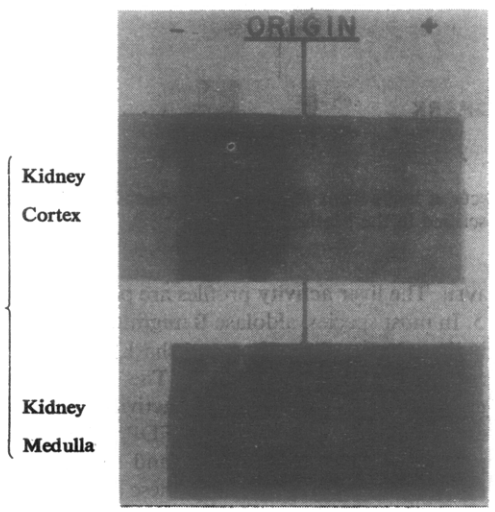


FIGURE 7: Zone electrophoretic resolution of aldolase activities in extracts of kidney cortex and medulla from the rabbit. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

As is evident by the profiles in Figure 5, low to moderate levels of aldolase A were also detected in many livers in addition to aldolase B. Few A–B hybrids were detected; thus, it is unlikely that A subunits are produced in significant quantities in the parenchymal cells which synthesize B subunits (Weber, 1965). Since aldolase A is probably tenfold more active than aldolase B in the activity assay (Rutter *et al.*, 1963b), the profiles emphasize deceptively the proportion of A activity. The A–C set was weakly apparent in chicken liver.

KIDNEY. The FDP/F-1-P ratios of the kidneys (Table I) suggest the presence of both aldolases A and B in this tissue. Members of the A–B hybrid set (Figure 6) were detected in most kidneys studied. As in liver, the single activity bands of human and monkey kidney presumably represent the activities of both aldolases A and B because of the low FDP/F-1-P ratios.

Aldolases A and B are detected in frog kidney. However, an A–B hybrid set is not evident in this tissue. Aldolases A and B were detected in shark kidney. In addition, several other activities were found. The relative mobilities and intensities of these activities suggest a complex distribution of the aldolase subunits in this tissue. Both the A–B and A–C (but not the B–C) hybrid sets were detected in chicken and turtle kidney.

The aldolase profiles of rabbit kidney cortex and medulla are shown in Figure 7. Aldolase A was the only activity found in medulla while members of the A–B hybrid set were detected in cortex. The FDP/F-1-P ratios also suggested that aldolase B was restricted to the cortex. The activity profiles of rat kidney tissues and the FDP/F-1-P ratios of the monkey kidney tissues also indicate this pattern of distribution.

TROUT AND SALMON. The tissue profiles of several

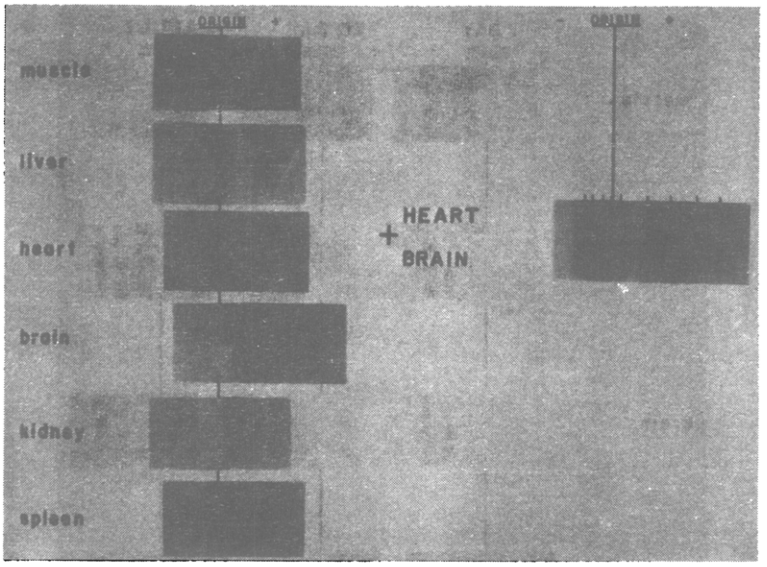


FIGURE 8: Zone electrophoretic resolution of aldolase activities in extracts of various trout tissues. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section. Profiles of the homologous salmon tissues were indistinguishable from these shown here. On occasion, slight staining at the origin was noticed, presumably due to enzyme activity associated with particulate matter.

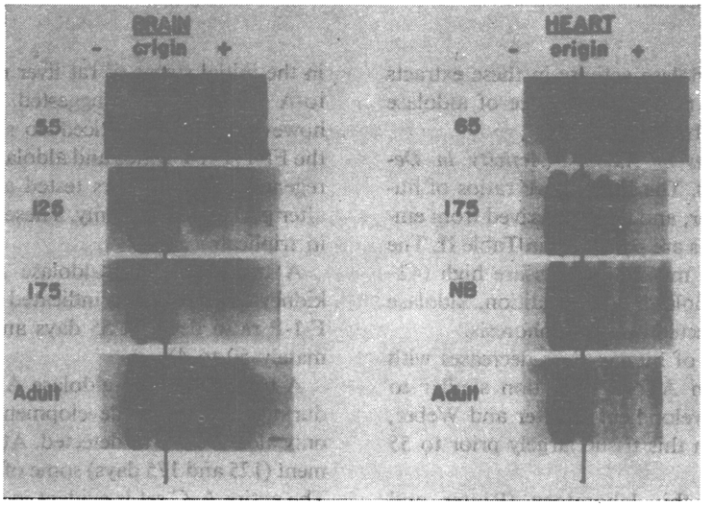


FIGURE 9: Zone electrophoretic resolution of aldolase activities in extracts of developing human brain and heart. The numbers refer to the approximate days after fertilization as estimated by crown-rump measurements (Iffy *et al.*, 1967). NB = newborn. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

trout populations (four generation inbred, wild steel head, and hybrid trout) and those of salmon were indistinguishable. As shown in Figure 8, the trout and salmon tissue profiles were qualitatively different from the profiles of other vertebrates studied.

By analogy with the other vertebrate systems, the single activity in skeletal muscle is presumed to be aldolase A. In addition to aldolase A, another more anodic band is found in liver which we surmise is aldolase B because of the low FDP/F-1-P ratios of these livers (2.4).

In addition to aldolases A and B, the presence of two additional parental forms in trout and salmon is inferred, since two different five-membered sets were detected in heart and brain, respectively. We have tentatively termed these forms aldolases "C" and "D." Coelectrophoresis of heart and brain extracts results in nine activities; thus one band (aldolase "C") appears to be common to both the heart and brain sets. At present it is not possible to determine the relationship of the trout aldolases, "C" and "D," to aldolase C of other vertebrates, because the properties of these enzymes have not been defined.

The predominant activity detected in kidney and spleen had a mobility equivalent to the common band of the heart and brain (aldolase "C"). However, upon prolonged incubation, additional members of the heart and brain hybrid sets were weakly apparent. Aldolase B activity was not observed in the kidneys of trout or

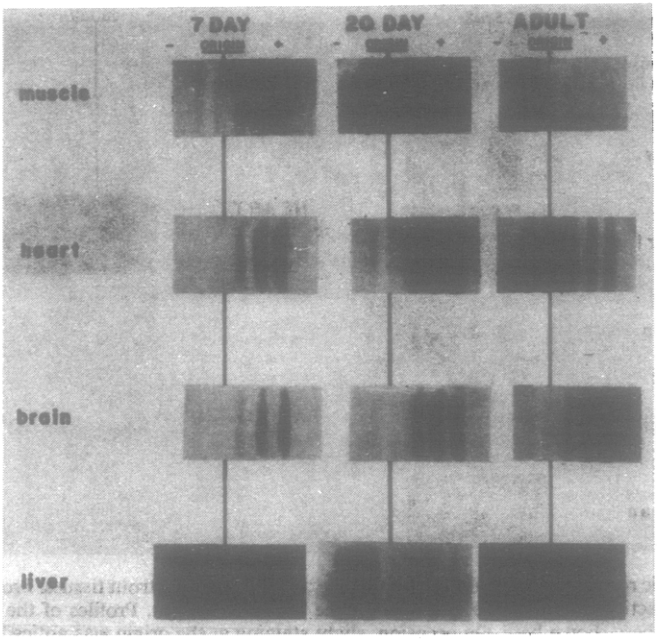


FIGURE 10: Zone electrophoretic resolution of aldolase activities in extracts of developing chicken muscle, heart, brain, and liver. The 7-day, 20-day, and adult profiles are shown. Procedures for tissue homogenization, electrophoresis, and activity staining are described in the Methods section.

salmon; however, the aldolase activity in these extracts was generally unstable; thus, the presence of aldolase B in this tissue has not been ruled out.

*Electrophoretic Profiles of Aldolase Activity in Developing Tissues.* HUMAN. The FDP/F-1-P ratios of human skeletal muscle, liver, and kidney derived from embryonic and adult sources are presented in Table II. The FDP/F-1-P ratios of all muscle extracts are high (42–50), characteristic of aldolase A. In addition, aldolase A was the only form detected by electrophoresis.

The FDP/F-1-P ratio of human liver decreases with development (3–1.2). An A-to-B transition similar to that seen in rat liver development (Rutter and Weber, 1965) probably occurs in this tissue largely prior to 55 days.

Earlier studies from this laboratory (Rutter and Weber, 1965) indicated an increase in FDP/F-1-P ratio in the initial stages of rat liver regeneration; thus, a B-to-A transition was suggested. In the present studies, however, we have noticed no significant differences in the FDP/F-1-P ratios and aldolase profiles between non-regenerating and livers tested at 1, 2, 3.5, and 8 days after partial hepatectomy. These studies were performed in triplicate.

A transition from aldolase A to B during human kidney development is indicated by the decrease in FDP/F-1-P ratio between 55 days and adult (from approximately 50 to 4).

A transition from aldolase A to the A–C set is seen during human brain development (Figure 9). At 55 days, only aldolase A was detected. At later stages of development (125 and 175 days) some of the A–C set was found. The entire A–C set is evident in the adult brain.

The A–C set was detected in all embryonic human hearts studied and in new-born heart (Figure 9). In con-

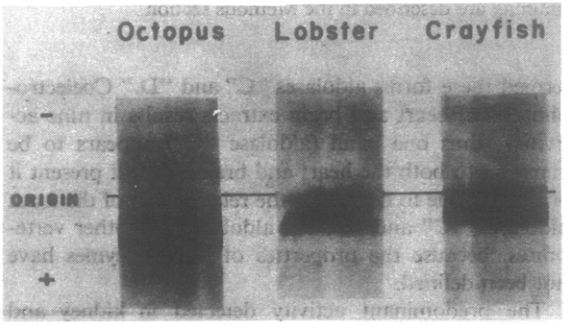


FIGURE 11: Aldolase activity profiles of octopus, lobster, and crayfish muscle extracts. The profiles of octopus heart and renal appendages and those of lobster and crayfish heart, brain, and hepatopancreas were indistinguishable from the corresponding muscle profiles. See text for special conditions of homogenization and electrophoresis.

TABLE II: FDP/F-1-P Ratios of Embryonic and Adult Human Muscle, Liver, and Kidney.[a]

| Tissue | 55 Day | 175 Day | Newborn | Adult |
|---|---|---|---|---|
| Muscle | 50 | 50 | 42 | 45 |
| Liver | 3.0 | 2.5 | 1.4 | 1.2 |
| Kidney | 50 | 10 | 7.4 | 4.0 |

[a] Embryonic age was estimated by crown-rump length (Iffy *et al.*, 1967). Conditions for tissue homogenization and determination of aldolase activity using FDP and F-1-P as substrate are described in the Methods section.

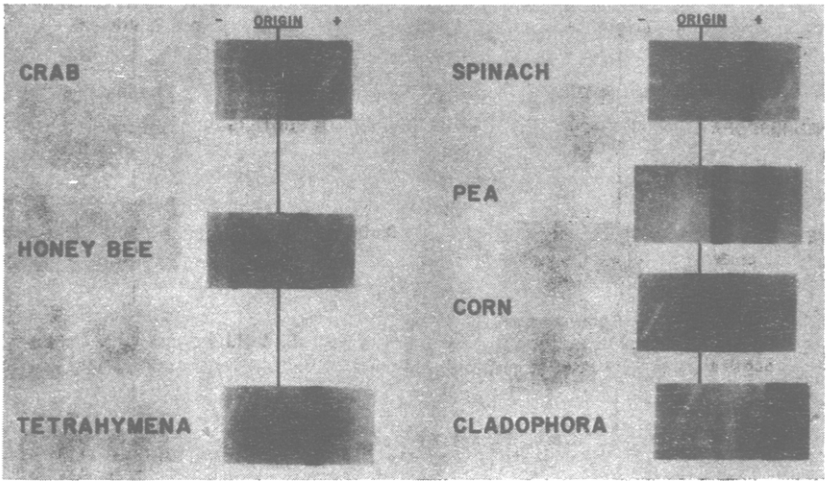


FIGURE 12: Zone electrophoretic resolution of aldolase activities in extracts of various invertebrate and plant species. The several anodic activities in pea and corn seedlings were weakly apparent. No differences were seen between head, thorax, and abdominal regions of the honey bee, between sonicated and nonsonicated spinach preparations, or between leaf, root, and stem regions of pea and corn seedlings. Procedures for homogenization, electrophoresis, and activity staining are described in the Methods section.

trast, aldolase A was the predominant activity detected in adult heart. Therefore, a transition from the A–C set to aldolase A probably occurs during human heart development and maturation.

CHICKEN. Aldolase C was the predominant activity detected in the early avian embryo (3 days). With development, a complete transition from aldolase C to aldolase A was seen in skeletal muscle (Figure 10). A transition from aldolase C to the A–C set was observed with chick heart ontogeny.

The embryonic and adult chicken brain profiles were similar. In this tissue, high levels of aldolase C were found at all stages of development.

The activity profiles of 7- and 20-day embryonic chick livers were similar to those of the adult. Aldolase B was detected in all of these livers. A C-to-B transition probably occurs earlier than 7 days since a decrease in FDP/F-1-P ratio from 17 to 2 was observed between 5 and 8 days (Rutter and Weber, 1965; Weber, 1965).

*Aldolase Activity Profiles of Invertebrates and Plants.* The aldolase activity present in invertebrate tissue extracts, particularly in extracts of hepatopancreas, was very labile. Activity profiles could be obtained by (1) homogenization of the tissues in 0.01 M Tris, 0.001 M EDTA, 0.003 M *o*-phenanthroline, 0.002 M phenylmethylsulfonyl fluoride, and 0.5 M sucrose (pH 7.5) which contained 0.07% $\beta$-mercaptoethanol, and (2) electrophoresis in the presence of 0.5 M sucrose. Using these expedients, the activity profiles and FDP/F-1-P ratios of several octopi (muscle, heart, and renal appendages), lobster, and crayfish (muscle, heart, brain, and hepatopancreas) tissues were obtained. The aldolase activities of all tissues of a given organism had the same electrophoretic mobilities (Figure 11). These activities could not be resolved by coelectrophoresis.

Although no differences in electrophoretic profiles were observed, the FDP/F-1-P ratios of the various tissues within a given species varied. For example, the ratio of crayfish muscle was high and that of hepatopancreas low (approximately 29 and 2.8, respectively). However, it was observed that the FDP/F-1-P ratios of hepatopancreas extracts decreased with time. It is well known that very low levels of carboxypeptidase activity reduce the FDP/F-1-P ratio of rabbit aldolase A from approximately 50 to 1 (Drechsler *et al.*, 1959). To test the possibility that the differences in FDP/F-1-P ratios of invertebrate tissues might be due to proteolytic modification of a single enzyme, crayfish muscle extract was incubated with hepatopancreas extracts at 25° for 1 hr. This treatment resulted in a drastic decrease in FDP/F-1-P ratio (29 to 1) and a severe inactivation (89%) of the muscle enzyme. Incubation of muscle extracts with distilled water under the same conditions resulted in es-

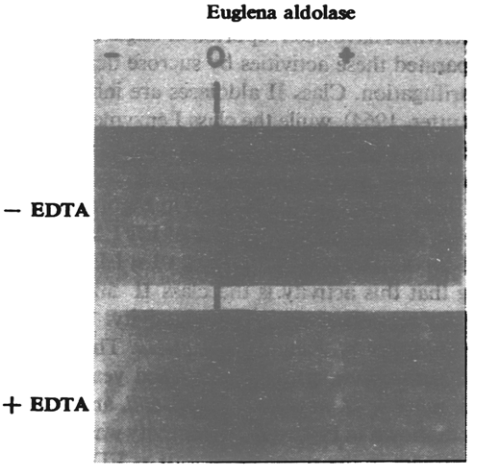


FIGURE 13: The resolution by zone electrophoresis of the class I and class II aldolases of *Euglena*. The normal procedures for electrophoresis and activity staining are described in the Methods section. For determination of the effect of EDTA, the strips were incubated on a 0.5% agar plate which contained 0.01 M EDTA at 4° for 10 min. The activity profile was then developed on an assay plate which contained 0.01 M EDTA.

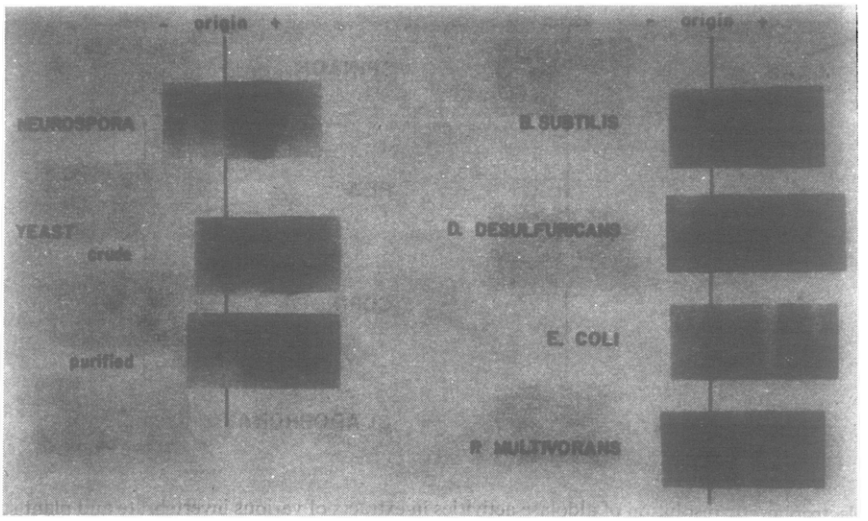


FIGURE 14: Aldolase activity profiles of various microorganisms. No activity bands appeared when electrophoresis and staining were performed in the presence of 0.01 M EDTA. Conditions for preparation of extracts, electrophoresis, and staining are described in the Methods section.

sentially no inactivation or decrease in FDP/F-1-P ratio. From these studies, it appears that the differences in FDP/F-1-P ratios of the invertebrate tissues studied are due to proteolytic modification and not to the presence of tissue-specific aldolase variants.

Multiple forms of aldolase were detected in crab muscle, but not in the head, thorax, or abdominal regions of the honey bee (Figure 12). Single activities were detected in *Tetrahymena*, in sonicated and nonsonicated spinach preparations, and in the green alga, *Cladophora*.

Multiple aldolase activity bands are evident in extracts of pea and corn seedlings (Figure 12). Similar profiles were obtained from leaf, root, and stem regions of the seedlings. The relative mobilities and intensities of these activities are not suggestive of typical hybrid sets.

*Euglena Aldolase Profile*. Both class I and class II aldolase activities have been reported in *Euglena*. Groves (1962) separated these activities by sucrose density gradient centrifugation. Class II aldolases are inhibited by EDTA (Rutter, 1964), while the class I enzymes are not. This difference in sensitivity to EDTA was used to identify the aldolases in *Euglena*. The electrophoretic resolution of these aldolases is presented in Figure 13. Two activities are detected in the absence of EDTA. The anodic band is completely inhibited by 0.01 M EDTA demonstrating that this activity is the class II enzyme. No heterogeneity was observed in either activity.

*Aldolase Profiles of Fungi and Bacteria*. The activity profiles of *Neurospora*, crude and purified yeast preparations, *B. subtilis*, *D. desulfuricans*, *E. coli*, and *P. multivorans* are shown in Figure 14. No activity was detected in these extracts in the presence of 0.01 M EDTA, thus demonstrating that all of these aldolases are no doubt class II enzymes. A single band of activity was detected in each organism.

In contrast to the above, the single aldolase activity in extracts of *M. aerogenes* was not affected by EDTA or other chelating agents. We recently have obtained this enzyme in a highly purified state; its properties resemble class I aldolases. These studies will be described in greater detail in a separate communication.

## Discussion

*The Phyletic Distribution of Multiple Aldolases*. CLASS I ALDOLASES. The present investigation suggests that aldolases A, B, and C (and, hence, their structural genes) are widely distributed in the vertebrates. Satisfactory identification of the parental aldolases A, B, and C has been made from their substrate specificities (FDP/F-1-P activity ratios), electrophoretic mobilities, tissue distributions, and, when necessary, specific immunological properties. Thus, even though the aldolases A and B of human tissues have indistinguishable electrophoretic mobilities, they can be identified by their selective inhibition by specific antibodies prepared against aldolases A and B of the rabbit.

Ohno *et al.* (1968) have recently reported that the salmoonid fishes, including salmon and trout, contain about twice the DNA content and twice the number of chromosome arms as compared with related families. They have concluded that these fish are polyploid, probably tetraploid. In support of this concept, Massaro and Markert (1968) have demonstrated four sets of lactate dehydrogenase isoenzymes in these fish whereas other closely related families contain only a single set. We have also observed numerous isoenzymic forms of glyceraldehyde 3-phosphate dehydrogenase in rainbow trout tissues and considerably fewer forms in perch (Lebherz and Rutter, 1967). In the present studies, we detected four parental aldolase types in trout and salmon, in contrast to three in all other vertebrates tested. If the salmonoids are, in fact, tetraploid, then duplicate genes for aldolases A, B, and C should be present (a total of six). The fourth parental form detected in the present experiments might have been derived from one of the other parental aldolase genes (C?). The distinctive tissue distribution of the fourth parental form, however, implies

that a specific ontogenetic function for this gene has developed in trout and salmon. Multiple genes for the other two aldolases were not detected. Clearly a more comprehensive study of the multiple forms of aldolases in the salmonoid fishes as well as in their close relatives would be desirable in order to further test the allotetraploidization hypothesis.

In contrast to the easily detected aldolase variants in vertebrates, multiple forms of class I aldolase were rarely observed in invertebrates. Multiple forms were, however, observed in crab muscle and in pea and corn seedlings, but five-membered sets were not detected and the molecular (and genetic) basis of the observed multiplicity remains to be defined. More detailed investigations of several tissues of the octopus, lobster, and crayfish have given no indication of distinct aldolase variants in these organisms. However, in confirmation of the earlier observations from this laboratory (Groves, 1962), differences in the FDP/F-1-P ratios were found in several tissues of these species. The ratio in the hepatopancreas was especially low; however, we noted that the FDP/F-1-P ratios of the aldolase activity of these extracts were somewhat variable and was always lowered by incubation. Furthermore, addition of muscle extracts containing aldolase A to hepatopancreas extracts results in a sharp decrease in the FDP/F-1-P ratio. The alteration of specificity of rabbit muscle aldolase A by treatment with carboxypeptidase is well known (Drechsler *et al.*, 1959). Therefore, the hepatopancreas must contain either carboxypeptidase-like activity or another proteolytic activity and we tentatively conclude that the low FDP/F-1-P ratios found in hepatopancreas are probably the result of an alteration of a single aldolase species found in all tissues. This point deserves more extensive experimental verification.

From this limited study of invertebrate tissues, we tentatively propose that the set of homologous aldolase genes originated, or at least were functionally consolidated, in the vertebrates, which have more extensive cellular and metabolic diversification than the lower animal forms. In addition, vertebrate cells usually have higher nuclear DNA content (McCarthy, 1965) and thus greater capacity for genetic duplication.

From the general knowledge of the relative structures of aldolases A, B, and C, it can be presumed that these genes are homologous in the evolutionary sense and are derived from a common class I aldolase gene ancestor (Rutter *et al.*, 1963b, 1968).

CLASS II ALDOLASES. In contrast to the readily detected class I variants of higher organisms, especially vertebrates, multiple forms of class II aldolases were not found in the fungi and bacteria studied. In previous studies (Groves, 1962) both class I and class II aldolases were detected in *Euglena*. The present studies give no evidence for multiple forms of either aldolase in *Euglena*.

A CLASS I BACTERIAL ALDOLASE? Until now, all of the bacterial aldolases studied in our laboratory (at least 25 species to date) are inhibited by EDTA and other chelating agents, and generally exhibit the properties of class II aldolases. In the course of the present investigation, however, we discovered that the aldolase of *M. aerogenes* was not inhibited by high levels of EDTA or other chelating agents. Preliminary studies on highly purified preparations of this enzyme suggest that it resembles class I aldolases. The relationship of this molecule to other class I enzymes and perhaps to the class II aldolases is currently under investigation.

*Aldolase Multiplicity and Subunit Structure.* The general occurrence of five-membered hybrid sets among the vertebrate species is predicted by the now well-established tetrameric structure of aldolases A, B, and C (Penhoet *et al.*, 1967). From these observations, we can conclude that the aldolases of all the vertebrate species examined are also tetrameric molecules.

The present studies, however, define only the minimal degree of aldolase multiplicity. It cannot be concluded, for example, that there are only three aldolase genes (four in trout and salmon) in all the vertebrate systems studied. We have detected multiple forms of aldolase A in bovine and swine muscle and Masters (1967) has observed multiplicity in the A band of mouse; thus, there may be frequent but not general duplicity of the major gene types. Slight structural variations, for example, allelomorphic differences in molecules, may be much more widespread, but detection of such minor molecular alterations is outside the scope of the present studies.

Yeast aldolase and probably other class II aldolases have a dimeric structure (C. Harris, R. Kobes, D. Teller, and W. J. Rutter, unpublished data). Thus, three-membered hybrid sets would be expected if two different subunits were synthesized in the same cell. In the present studies, we have not detected multiple forms in any cells containing class II aldolases.

*Selective Tissue Distribution of the Vertebrate Aldolases.* As shown in Table III, there is a generally segregated tissue distribution of the parental aldolases. Aldolase A was found in most tissues studied; aldolase B was restricted to liver and kidney; the tissue distribution of aldolase C varied somewhat more widely.

The cellular distribution of aldolase B agrees with the proposed role of this enzyme in gluconeogenesis and fructose metabolism (Rutter *et al.*, 1963a). The metabolism of fructose *via* fructose 1-phosphate occurs primarily in the liver. Both liver and kidney cortex are rich in gluconeogenic activity (Benoy and Elliott, 1937; Krebs *et al.*, 1963). Our finding that aldolase B is present in the rabbit kidney cortex and was not detected in the medulla agrees with the proposed function of this enzyme.

There is as yet no known catalytic basis suggesting a separate physiological function for aldolases A and C. Since aldolase C is generally present among the vertebrates and has a specific tissue distribution in each vertebrate organism tested, we surmise that the gene, and hence, its product, has a specific physiological function. The enzyme could, for example, have as yet undiscovered catalytic properties. Alternatively it might have a unique intracellular distribution which is related to a specific function (Penhoet *et al.*, 1966). It is also possible that the physiological basis for its existence may be found within yet-to-be defined ontogenetic mechanisms.

*Regulation of Synthesis of the Class I Aldolases.* Little is currently known about the regulation of the verte-

TABLE III: Tissue Distribution of Aldolases A, B, C, and D in Vertebrates.

| | Muscle | Spleen | Heart | Brain | Liver | Kidney |
|---|---|---|---|---|---|---|
| Human | A | A | *A*–C | A–C | A, *B* | A–B |
| Monkey | A | A | *A*–C | A–C | A, *B* | A–B |
| Bovine | A | *A*–C | A | A–C | A, *B* | A–B |
| Swine | A | *A*–C | *A*–C | A–C | A, *B* | A–B |
| Rat | A | A | A | A–C | A, *B* | A–B |
| Rabbit | A | A | A | A–C | A, *B* | A–B |
| Chicken | A | A–C | A–C | A–*C* | A, *B* | A–B, A–C |
| Turtle | A | A | *A*–C | A–*C* | A, *B* | A–B, A–C |
| Frog | A | A | A–C | A–C | A, *B* | A, B |
| Perch | A | A–C | *A*–C | A–*C* | A, *B* | A–*B*, A–C |
| Shark | A | A | A–C | A–C | A, *B* | A, *B* |
| Trout, salmon | A | "C" | A–"C" | "C–D" | A, *B* | "C"? |

[a] The appropriate hybrid sets are designated by an en dash between the parental aldolase types (*i.e.*, A–B, A–C, and "C–D"). The dominant subunit in the hybrid sets is in italics. The assignment of aldolases "C" and "D" in trout and salmon tissues is tentative, and uncertainty (?) of the presence of aldolase B in trout and salmon kidney is indicated.

brate aldolases. From the segregated tissue distribution of these enzymes, it can be concluded that they are independently regulated. This conclusion is strengthened by the observations in a number of systems of transitions from synthesis of predominantly one subunit type to another during embryological development. The detection of transitions from aldolase A to B, A to C, and C to A in the present studies supplements the earlier observations in this laboratory of A-to-B and A-to-C transitions in rat liver and brain, respectively (Weber and Rutter, 1964; Rutter *et al.*, 1963a; Rutter and Weber, 1965; Weber, 1965; W. A. Susor and B. Luppis, unpublished). Many of our experimental observations have been confirmed by others. Rensing and associates (1967) have, for example, detected the aldolase A-to-B and A-to-C transitions in rat liver and rat brain, respectively. Herskovitz *et al.* (1967) have observed the C-to-A transition in chick muscle.

In earlier studies (Rutter *et al.*, 1963b) we reported that an increase in FDP/F-1-P ratio occurred during liver regeneration, thus suggesting a possible B-to-A transition associated with the initial stages of regeneration. In the present more definitive studies, we have found no consistent alteration in FDP/F-1-P ratios or in the electrophoretic activity profiles between normal and regenerating liver. Thus, the earlier results were probably caused by the adventitious presence of different proportions of erythroid or other cells containing aldolase A (Rutter and Weber, 1965; Rutter *et al.*, 1963a). Even though a B-to-A transition was not observed during liver regeneration, B-to-A transitions do indeed occur in certain liver hepatomas. We have reported that slow-growing, minimal deviation hepatomas had low FDP/F-1-P ratios, characteristic of aldolase B from normal liver, whereas more rapidly growing invasive hepatomas invariably contained aldolase A (Rutter *et al.*, 1963a). More recently, Matsushima and his colleagues (1968) have confirmed these results and showed that the distribution of aldolases A and B differed between normal and tumor cells; thus there is a modified synthesis of A and B subunits between normal and variant liver.

In the studies available to date, the ontogenetic transitions have involved predominantly a shift in the synthesis from A to either B or C subunits. This would imply that the major aldolase present in early embryonic cells is aldolase A and that the synthesis of aldolase B or C is initiated with cytodifferentiation. On the other hand, a number of C-to-A transitions have been detected especially in chicken. Moreover, we have noted that the early chicken embryo (3 days) contains aldolase C. These observations make it of special interest to determine the aldolase type(s) found in the earliest stages of embryogenesis in mammalian and avian species. An interchangeability of aldolases A and C during early development might indicate a functional equivalence of these two molecules.

We have never observed B–C hybrids in tissue extracts. Since such hybrids can be readily produced by reversible dissociation of a mixture of aldolases B and C (Penhoet *et al.*, 1966) we conclude that aldolases B and C are rarely, if ever, synthesized in the same cell.

Two general mechanisms for the regulation of the three homologous aldolases A, B, and C can be proposed. The first mechanism essentially establishes constitutivity. During the determination of differentiation of a given cell type, a specific degree of regulation of the aldolase genes would be established at the genetic level and perpetuated in that cell and, perhaps, its progeny. This mechanism of regulation would be expected to be rather independent from short-term metabolic modulations. A second mechanism would invoke a direct role of a metabolite effector and hence presumably would be dependent on the perpetuation of a constant meta-

bolic state. Thus, the synthetic transition from synthesis of one subunit type to another in embryological development would be the secondary result of a metabolic transition. This mechanism would perhaps allow a more dynamic response to physiological alterations.

The present studies, as well as previous ones from this and other laboratories, provide a number of systems which can be used to study the regulation of synthesis of the aldolases. The demonstration that the A–B transition can be obtained *in vitro* (Rutter and Weber, 1965; Weber and Rutter, 1964; Weber, 1965) suggests that other transitions may similarly be studied in simplified systems and thus the mechanisms of regulation of these three molecules is now susceptible to definitive experimentation.

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