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THE ONTOGENY OF MAMMALIAN FRUCTOSE-1,6-DIPHOSPHATE ALDOLASE

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SUMMARY

1. The ontogeny of aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7) multiple forms has been studied in four mammalian species, in order to clarify comparative aspects of the control of synthesis, structural inter-relationships and biological significance of the enzyme.

2. Developmental progressions of the aldolase multiple forms were observable in all the tissues followed, with the sole exception of brain. The stability of the isoenzyme pattern in this tissue, paradoxically, may indicate that the primary biological role of aldolase C is not neurally oriented.

3. In the early developmental stages of each animal, the relative contributions of aldolase A- and C-type activities were constant. The ratios of these activity types were species specific and serve to emphasize the developmental importance of aldolase C.

4. Overall, the ontogenetic properties of this enzyme and the presence of five-membered isoenzyme sequences provide support for the conception of a tetramer structure of aldolase and the involvement of three independent structural genes in the control of synthesis of the aldolase multiple forms in mammalian species.

INTRODUCTION

Fructose-1,6-diphosphate aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7; fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, 4.1.2.13) is an essential component of the glycolytic pathway and a common cellular constituent of animals, plants and micro-organisms. The properties of the vertebrate enzyme, in particular, have been studied extensively, with several groups of investigators having contributed to an understanding of the comparative enzymology and structural characterization of this enzyme[1-4].

Until recently, however, the available data were generally interpreted as indicating that aldolase possessed a trimeric structure and existed in vertebrate

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tissues as two main forms (aldolase A and B). It is now known that this enzyme exhibits a far more extensive heterogeneity than had been considered previously[5-9], and this realization has led to a reappraisal of many of the previous conceptions of this enzyme. Aldolase is now considered to be a tetramer rather than a trimer, for example, and the multiplicity of the enzyme seems to be explicable on the basis of five-membered hybridization sequences between three parental forms[10,11].

Considerable uncertainty remains at present, though, in regard to the details of subunit composition, gene control and physiological significance of the multiple forms of this enzyme[12,13]. The present communication was prompted by the recognition of this need for clarification and initiated with the knowledge of the significant contributions made to similar problems in other isoenzyme systems by developmental studies[13-16]. In this investigation, the ontogeny of aldolase multiple forms has been described in four mammalian species and correlated with the establishment of tissue identity and with the heterogeneity and control of synthesis of this enzyme.

METHODS

Fresh tissues were excised, frozen and stored at $-10°$ until required for analysis. Homogenates of these tissues were prepared in 2 vol. of 0.01 M Tris–HCl buffer (pH 7.5), which contained EDTA (0.001 M) and β-mercaptoethanol (0.01 M). The homogenates were then centrifuged at 100 000 $\times$ g and 2° for 60 min, after which the supernatant extracts were removed and analysed.

Zone electrophoresis of these tissue extracts was carried out in starch gels at 4° with a current density of 3 mA/cm (ref. 22). After 15 h in a horizontal gel at pH 7.0, or 16 h in a vertical gel at pH 9.0, the gels were sliced and stained for aldolase activity by a modification of the method of DEWEY AND CONKLIN[17]. Agar (1.5%) was dissolved in pyrophosphate buffer (0.05 M, pH 8.5), and 50 ml of this solution were maintained at 55° in the dark, while the following reactants were added with mixing: 1 ml of 0.3 M sodium arsenate, 2 ml of 0.2 M fructose 1,6-diphosphate, 1 ml of NAD (30 mg/ml), 1 ml of thiazolyl blue (10 mg/ml), 0.25 ml of phenazine methosulphate (10 mg/ml) and 0.4 ml of glyceraldehyde-3-phosphate dehydrogenase. This mixture was then poured over the starch gels and allowed to develop in the dark for 3 h at 25°. The resulting zymograms were scanned in an integrating densitometer and the percentage of activity type (aldolase A, B or C) calculated by a methodology which has been described previously[18].

Tissue-extract supernatants were assayed for aldolase activity by the method of RICHARDS AND RUTTER[19]. The rate of the coupled reaction was estimated by measurements at 340 mμ in a Zeiss spectrophotometer at 25°. Enzyme activity was calculated as international units per l, and the specific activity was determined by relation to the protein concentrations, estimated by the method of LOWRY *et al.*[20], with crystallized bovine serum albumin for standards.

RESULTS

The zymograms obtained from the tissues of the adult guinea pig, hamster, rat and rabbit are illustrated in Fig. 1. It may be seen that the isoenzyme patterns for skeletal muscle are similar for each species, with the activity localized in a single band

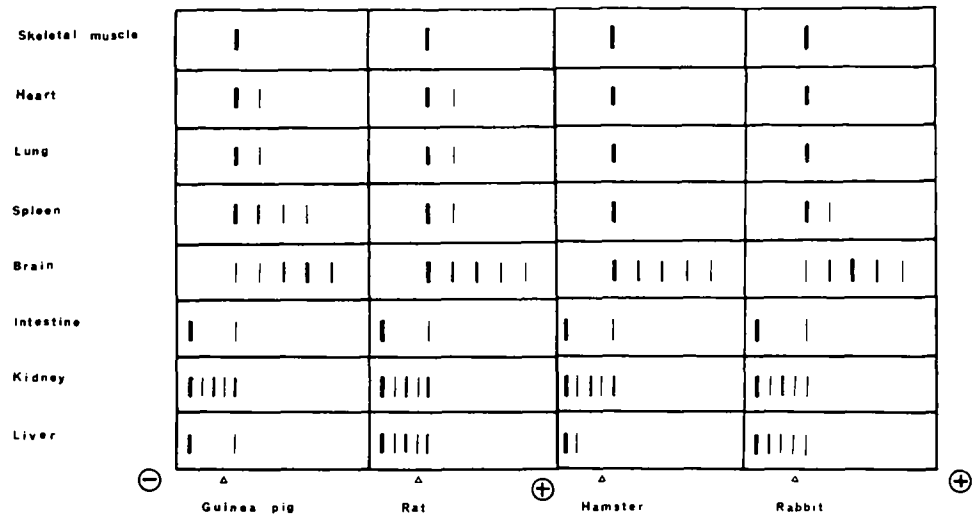

Fig. 1. Diagrammatic representation of the aldolase zymograms from adult tissues of the guinea pig, rat, hamster and rabbit.

which migrates only a short distance from the origin under the experimental conditions. By analogy with the established properties of aldolase in these and other species, this band may be designated as aldolase A, or aldolase-5 (refs. 1, 32). Heart, lung and spleen vary little from this muscle pattern, although in the guinea pig and rat tissues some further anodic banding is evident.

In the case of brain, all these species show an extra 4 anodic bands with an activity distribution of an approximately binomial type. The relative emphasis of this distribution varies somewhat between the species, with an increasing anodal expression in the order hamster, rat, rabbit, guinea pig. These band positions have been referred to as aldolases-1, -2, -3 and -4 in previous publications[7,11]. Once again, by analogy with the demonstrated interrelationships in other species, these bands are considered to correspond to AC hybrids (aldolase-2, -3, -4) and the parental C forms (aldolase-1) of aldolase. In order to verify this identity, aldolases-1 and -5 were eluted from starch gels of each species and subjected to dissociation–reassociation treatments[21]. The electrophoretic pattern of aldolases-1 and -5, singly, were not altered by this procedure, but when combined, the full set of isoenzymes (aldolases 1, 2, 3, 4, 5) resulted, with the predominant activity in the median bands. A mixture of aldolases-1 and -5 from rabbit tissues, for example, produced a pattern after dissociation-reassociation which was similar to that in adult rabbit brain (Fig. 1).

With liver and kidney, additional heteromorphs are evident on the cathodal side of the origin. Liver, for example, possesses the major region of activity on the cathodal side of aldolase-1, and kidney and intestine also display this type of activity. Known characterization in other species, and the substrate activity ratios of these forms, point to an identity with aldolase B and the AB hybrids[8,19] (Fig. 1).

Additional bands to those described are observable in the kidney and liver of these species, but are demonstrable as non-specific dehydrogenases by their preferential staining in the absence of added substrate. It is of interest to note that heterogeneity

is observable in some of these non-specific positions, also. Hamster shows 3 regularly spaced regions of this type of activity.

Further properties of the aldolase in the tissues of the adult animal may be derived from Table II. It has been demonstrated previously that aldolases A, B and C possess different relative specificities towards fructose 1,6-diphosphate and fructose 1-phosphate[8,11], and these ratios provide a useful complementation to the electrophoretic data for these animals. In general, the substrate activity ratio for aldolase C is indicated as intermediate to the values for aldolase A and B in any one of these species. There appears to be a greater inter-species variation of the substrate ratio for C, however, than for aldolase A or B.

On the basis of specific-activity estimations, skeletal muscle, heart, brain, liver and kidney are indicated as the major enzyme sources.

During the maturation and embryonic development of these mammals, a considerable redistribution of activity occurs among the aldolase isoenzymes, and these changes are illustrated in Tables I and II and Figs. 2, 3 and 4. In all of these mammalian species, the early embryonic enzyme contains appreciable proportions of aldolase-C activity as well as the A-type. Aldolase A becomes the predominant activity type for skeletal muscle and most other tissues during the major part of maturation, but in brain, aldolase C remains present in foetal proportions throughout development.

TABLE I

CHANGES IN SUBSTRATE ACTIVITY RATIOS DURING DEVELOPMENT

Relative activities against fructose 1,6-diphosphate and fructose 1-phosphate.[19] Prenatal age is expressed as time of gestation. Post-natal age (+) is indicated as time after birth.

Age	*Muscle*	*Brain*	*Heart*
Guinea pig			
5 weeks	40	40	40
7 weeks	45	40	45
9 weeks	50	38	45
+1 week	50	40	50
adult	55	40	55
Hamster			
9 days	45	45	45
13 days	47	45	45
16 days	50	45	45
+1 day	50	45	50
adult	50	45	50
Rat			
12 days	43	43	43
16 days	43	43	43
22 days	45	43	45
+7 days	45	43	45
adult	45	43	45
Rabbit			
10 days	40	35	35
15 days	45	35	40
25 days	50	35	50
31 days	50	35	50
adult	55	35	55

TABLE II

PROPERTIES OF ALDOLASE IN ADULT AND FOETAL TISSUES

Pre-natal age is expressed as time of gestation. Post-natal age(+) is indicated as time after birth. Substrate activity ratio: relative activities against fructose 1,6-diphosphate and fructose 1-phosphate.[19] Specific activity: international units of activity per mg protein.

Tissue	*Age*	*Substrate activity ratio*	*Specific activity*
Guinea pig			
Liver	5 weeks	1.4	48
	7 weeks	1.2	45
	9 weeks	1.1	46
	+1 week	1.1	45
	adult	1.1	42
Kidney	7 weeks	2.8	50
	9 weeks	2.2	60
	+1 week	2.0	65
	adult	2.0	65
Lung	adult	50	80
Spleen	adult	47	50
Intestine	adult	2.8	20
Whole embryo	3 weeks	40	30
Hamster			
Liver	13 days	1.1	48
	16 days	1.1	50
	+1 day	1.1	50
	adult	1.1	40
Kidney	16 days	2.0	36
	+1 day	2.0	35
	adult	1.9	30
Lung	adult	50	58
Spleen	adult	48	45
Intestine	adult	2.9	20
Whole embryo	adult	45	25
Rat			
Liver	12 days	1.2	45
	16 days	1.2	50
	22 days	1.1	50
	+7 days	1.1	38
	adult	1.1	28
Kidney	22 days	2.4	40
	+14 days	2.2	38
	adult	2.2	24
Lung	adult	43	35
Spleen	adult	43	30
Whole embryo		43	30
Rabbit			
Liver	15 days	1.4	120
	25 days	1.2	160
	31 days	1.2	160
	adult	1.0	85
Kidney	25 days	3.0	40
	31 days	2.8	40
	adult	2.5	65
Lung	adult	50	40
Spleen	adult	50	35
Whole embryo	10 days	35	20

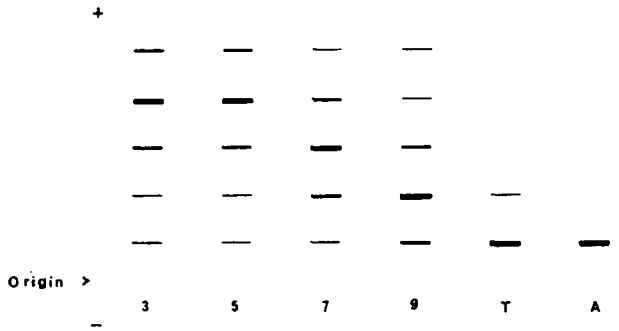

Fig. 2. Patterns of aldolase activity in samples of skeletal muscle from guinea pigs at different stages of development. The numbers refer to the time of gestation (weeks). T refers to a term (newly born infant) and A to an adult specimen.

Aldolase-B activity becomes evident in kidney and liver at an early stage of functional differentiation.

Changes in the specific activity of maturing tissues (Table I, Fig. 4) are greatest in skeletal muscle at the immediate post-natal stage. Heart also shows appreciable increases in specific activity, generally occurring at an earlier stage than in skeletal muscle. With liver, a decline in specific activity is evident in the post-natal period of all these species.

The response to dialysis of some representative tissues of these species are presented in Table III. The aldolase activities of embryonic tissues were not significantly altered by this treatment, and only slight differences in fructose 1,6-diphos-

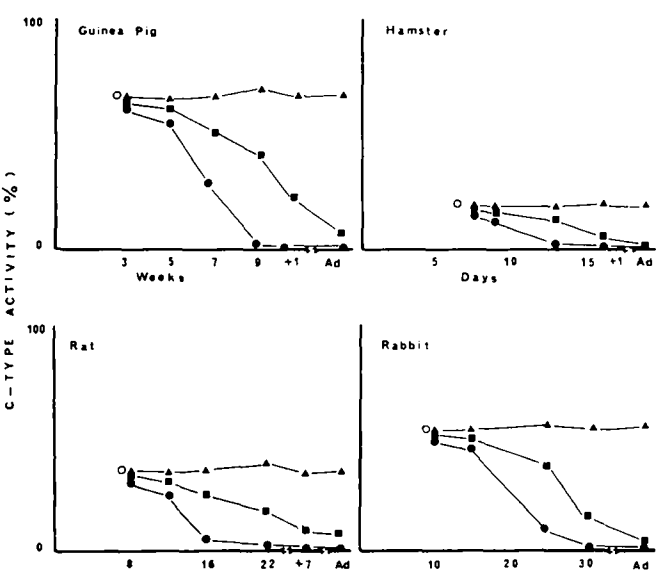

Fig. 3. Alteration in the type of aldolase activity during development. ■, Heart; ▲, brain; ●, skeletal muscle; ○, whole embryo.

TABLE III

THE INFLUENCE OF DIALYSIS AND MIXING ON THE ALDOLASE ACTIVITIES IN MAMMALIAN TISSUES

The supernatant fractions from tissue extracts (see METHODS) were dialysed for 16 h at 4° against Tris–HCl buffer (0.01 M, pH 7.4) containing 0.001 M EDTA and 0.01 M β-mercaptoethanol. For the admixture experiments, equal volumes of the appropriate adult and embryonic supernatants were used. Assay procedures are described in METHODS section.

Tissue	*Specific fructose 1,6-diphosphate cleavage activity*		*Substrate activity ratio*	
	Before dialysis	*After dialysis*	*Before dialysis*	*After dialysis*
Guinea Pig				
Adult brain	28.3	29.1	40	41
Adult heart	27.2	28.2	55	57
Adult muscle	30.4	30.4	55	55
Whole embryo (20 days)	20.1	20.0	40	40
Adult brain *plus* embryo	24.1			
Adult heart *plus* embryo	23.9			
Adult muscle *plus* embryo	25.0			
Hamster				
Adult brain	23.7	22.6	45	43
Adult heart	27.5	27.5	50	50
Adult muscle	26.4	26.0	50	50
Whole embryo (7 days)	22.3	20.9	45	43
Adult brain *plus* embryo	23.0			
Adult heart *plus* embryo	24.8			
Adult muscle *plus* embryo	24.1			
Rat				
Adult brain	21.4	21.0	43	42
Adult heart	22.7	23.2	45	46
Adult muscle	29.2	29.2	45	45
Whole embryo (7 days)	19.8	19.2	43	42
Adult brain *plus* embryo	20.9			
Adult heart *plus* embryo	21.9			
Adult muscle *plus* embryo	24.4			
Rabbit				
Adult brain	25.2	25.0	35	35
Adult heart	22.8	22.8	55	55
Adult muscle	28.6	28.4	53	53
Whole embryo (10 days)	20.2	20.0	35	35
Adult brain *plus* embryo	22.9			
Adult heart *plus* embryo	22.0			
Adult muscle *plus* embryo	24.2			

phate cleavage activity were noticed in the adult tissues. Mixing of the supernatant fractions from embryonic and adult tissues of these, again caused little deviation from the mean of the separate activities. Livers were not included in these comparisons because of the presence of significant peptidase activity in these homogenates: on the other hand, none of the tissues listed in Table III exhibited appreciable proteolytic ability.

In Fig. 5, the proportions of activity type are compared in the aldolase and lactate dehydrogenase of early vertebrate embryos. The developmental stage in all

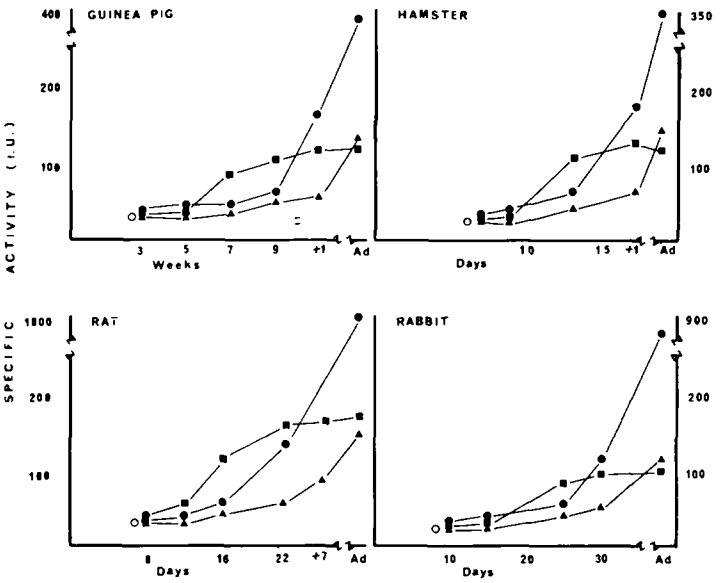

Fig. 4. Alteration in the specific activity of aldolase during development. ■, Heart; ▲, brain; ●, skeletal muscle.

these species corresponds to the initiation of functional differentiation, and the lactate dehydrogenase data were derived from the results of FIELDHOUSE AND MASTERS[28].

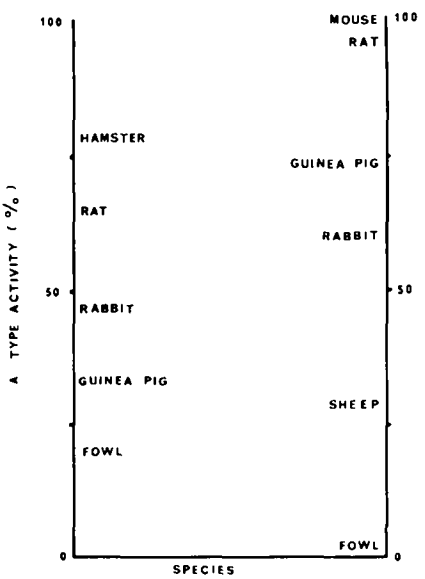

Fig. 5. The type of aldolase (left-hand scale) and lactate dehydrogenase (right-hand scale) activity in the early embryos of vertebrate species.

DISCUSSION

Although aldolase-C has been shown to possess a broad tissue distribution in avian species[7], the evidence in this and other studies indicates that mammalian C-type activity is largely restricted to the nervous tissue of this class of animal[8,10]. This localization allows little margin for the display of organ specificity in the distribution patterns of mammalian aldolase isoenzymes, and only brain, liver and kidney of these species can, in general, be said to exhibit distinctive homologous patterns of aldolase multiple forms.

It is of interest, though, that the mammalian tissues other than brain which do display some C-type activity (*e.g.* heart, spleen and lung) are just those which evince the highest C-type activity in avian species. With lactate dehydrogenase (which displays many similarities of heterogeneity to the aldolase AC forms), it has been pointed out that differences in tissue isoenzyme patterns between species may often be generalized as a shift of emphasis in favour of a particular sub-unit type, *i.e.* the factors causing differential gradations of sub-unit synthesis between species may be considered as influencing the whole animal rather than being restricted to individual tissues only[23]. It would seem from the avian-mammalian comparison that similar arguments may well apply to the control of aldolase A- and C-type syntheses, as well. Aldolase B, in contrast, appears to be markedly tissue specific in its distribution although some variations in the A-B-type distribution in liver are seen amongst the four mammalian species.

Physiological roles for aldolases A and B have been proposed by previous investigators on the basis of the substrate specificities and tissue distribution of these enzyme types[24]. As yet, however, no distinctive biological role for aldolase C has become evident, although the high content in brain has caused speculation as to a neural role for this form of the enzyme. Further insight into this problem may be provided by the developmental data in this communication. It should be noted, for example, that the behaviour of brain is unusual in that this is the only tissue in all the species examined which displays inappreciable maturative alterations in the patterns of aldolase heterogeneity (Fig. 3). Again, this situation persists in spite of the striking changes of carbohydrate metabolism which are known to occur in the brains of developing mammalian foetuses[25].

One explanation which is consistent with these experimental facts and with modern conceptions of protein synthesis[26], is that the early establishment of a blood–brain barrier in vertebrates may restrict the passage to the brain of effector substances having a directive influence on the type of aldolase synthesis. Hence the localization of aldolase C in brain would not necessarily reflect a special physiological suitability of this enzyme type to a neural environment, but rather an extrinsic happenstance of embryonic development. In this connection, it would seem to be important that the generality of this developmental behaviour be tested with other vertebrates, and this aspect is presently under investigation in this laboratory.

Another noteworthy feature of the ontogenetic sequences of aldolase heteromorphs in these species is that, in the many tissues of these animals, both foetal and post-natal, where the isoenzyme sequences have been examined, the experimental data are consistent with the existence of five-membered sets of multiple enzyme forms (*i.e.* an AC set and an AB set). Such a regular stoichiometry in these and other species,

along with the equidistant spacing in each set of isoenzymes, is considered to provide strong biological confirmation of the tetrameric structure of aldolase[10,11]. In addition, the inter-relationships of these mammalian aldolase forms emphasize the desirability of a modification in the classification of aldolase presently recommended by the Enzyme Commission[33]. Of the two aldolases listed, EC 4.1.2.7 clearly refers to the liver enzyme (aldolase B) while EC 4.1.2.13 includes aldolase A, and presumably aldolase C, as well as the aldolases of bacteria, yeasts and fungi[33,34]. Instead of this arrangement, there would appear to be a cogent argument in favour of reserving one of these classifications (*e.g.* 4.1.2.7) for vertebrate aldolases and enzymes with similar properties, and the other for the aldolases of bacteria, yeasts and fungi. It is now well established, for example, that the vertebrate aldolases are a family of inter-related proteins with many similar properties, whereas markedly different characteristics (*e.g.* metal requirements, molecular weights, probable catalytic mechanisms) have been established for the aldolases of bacteria, etc.[1,7,8,11]. The difficulties of enzyme and isoenzyme classification would appear to be unnecessarily compounded when separate numeration is required within a hybridization sequence, on the one hand, but not between grossly dissimilar proteins, on the other.

In terms of genetic expression, the developmental changes in these animals may be summarized as representing a constant relative expression of the aldolase A and C genes during the initial stages of the functional differentiation of these species, followed by increased relative expression of the A gene during the maturation of most tissues. The B gene is activated at a later stage than the A and C genes but is pre-eminent in liver and kidney during the majority of the developmental stages. Since the early embryonic patterns of lactate dehydrogenase have provided important information on the control of isoenzyme synthesis previously[13,14,27,28], a comparison of the aldolase and lactate dehydrogenase situations at this stage of differentiation is relevant and useful. While it was originally believed that the initial enzyme synthesis of lactate dehydrogenase was monotypal, with avian species producing heart-type lactate dehydrogenase, and mammalian species the muscle form of this enzyme at this stage of development, it has recently been demonstrated that individual vertebrates exhibit relative expressions of the A and B genes which range over the entire scale of intermediate possibilities. This situation shows similarities to the aldolase position. With this enzyme, too, the early embryos of all reported species show constant relative expression of two aldolase genes (A and C) (Fig. 5). Although the spread of initial, percentages of aldolase C is not as great as that recorded for lactate dehydrogenase B, it is clear that synthesis of aldolase also is not initially monotypal and therefore not closely analagous to the compensatory interdependence of the haemoglobin synthetic scheme[29]. It is evident also that the relative percentages of aldolase C at this stage of development do not bear a proportional relationship to those for lactate dehydrogenase B, nor to the degree of oxygenation of these embryos[23,28,30], hence, neither the directive influences of lactate dehydrogenase synthesis nor the degree of oxygenation appear to bear a direct epigenetic relationship with aldolase synthesis. These values also establish the developmental importance of C-type aldolase activity, and demonstrate that the primary embryonic form of aldolase in mammalian species is not necessarily the muscle-type enzyme (aldolase A), as has been suggested previously[31].

The specific activity data and dialysis experiments in these species provide further information on this control of aldolase synthesis in mammals. Dialysis and

admixture studies, for example, indicate that no appreciable influence of inorganic cofactors or polypeptide inhibitors is exerted on aldolase activity in the developing tissues of these species. The marked post-natal increase in muscle aldolase activity which occurs in all these animals studied, then, points to a derepression of A-type synthesis and independent regulation of the A and C genes, and similarly, B-gene activity is indicated as independent by the sequential specific activities in kidney and liver.

In summary, the evidence provided by the study of the ontogeny of mammalian aldolase serves to confirm and strengthen previous indications that the synthesis of aldolase in most vertebrate tissues is governed by three independent structural genes (A, B and C).

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