BBA 66082

STUDIES ON ISOZYMES OF FRUCTOSE-1,6-DIPHOSPHATE ALDOLASE DURING AMPHIBIAN DEVELOPMENT

II. CHARACTERIZATION OF ISOZYMES IN THE EGG OF *RANA PIPIENS*

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(Received October 20th, 1969)

SUMMARY

1. The types of fructose-1,6-diphosphate aldolases (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7; fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) found in the unfertilized frog egg, as well as in early stages of development, have been investigated to gain insight into the biological significance of aldolases of the A, B, and C types in early embryogenesis.

2. The relative electrophoretic mobilities of the isozymes from egg and adult frog tissues indicated that five-membered hybrid sets of the A–C types are present in the unfertilized egg.

3. The five isozymes in egg and in brain have been separated by chromatographic procedures and their properties compared. The substrate specificities, electrophoretic mobilities, and immunological properties of the individual isozymes from both sources were comparable and differed significantly from those of frog liver aldolase (type B). Dissociation and reassociation of a given hybrid isozyme from egg led to the formation of the other four isozymes of aldolase.

4. These studies show that A and C type aldolases, together with their hybrids, are present in the unfertilized frog egg.

5. No marked changes in the relative proportions of the A–C isozymes were observed in embryos up to hatching at which time muscle aldolase (type A) increased.

INTRODUCTION

On the basis of molecular and catalytic properties, three parental forms (types A, B and C) of 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) have been detected in tissues of a variety of vertebrate species. Work in this area, which has been carried out in several laboratories, has been reviewed recently by

RUTTER *et al.*[1]; additional references may be found in a recent paper by LEBHERZ AND RUTTER[2]. Five-membered A–B and A–C hybrid sets of aldolases have been demonstrated in certain vertebrate tissues (*cf.* ref. 1) or can be formed *in vitro* by the combination of the respective parental subunits[3]. These and other data support the conclusion that the aldolase molecule has a tetrameric structure[4–6].

Several studies have been carried out on aldolase isozymes during organogenesis. A transition from aldolase A to B and from A to C has been observed in developing rat liver and brain, respectively[7,8]. In the human, transitions from aldolase A to B in liver and kidney and A to C in brain have been reported[2]. On the other hand, MASTERS[9] has reported that there are appreciable quantities of aldolase C as well as A in most embryonic tissues of the rat, rabbit, guinea pig and hamster. Similarily, transitions from a predominance of aldolase C to A have been observed in chicken muscle and heart[2,10] and in human heart[2] with maturation.

Studies on isozymes in the developing amphibian are under investigation in this laboratory. This system was chosen since the unfertilized frog egg is readily available in the quantities needed for studies of this type and, in addition, possible changes in the isozyme patterns of this primordial cell following fertilization and during development can be investigated. Comparative studies on the electrophoretic patterns of aldolase isozymes in crude extracts of amphibian tissues, as well as fructose-1,6-P_2/fructose-1-P activity ratios of such preparations, were reported earlier from this laboratory[11]. The results indicated that the aldolases of the unfertilized frog egg are of the A and C types. Further studies on the immunological and kinetic properties of the individual isozymes found in frog egg and brain, which extend and confirm our earlier results, are presented here.

METHODS

Substrates, enzymes and other chemicals

NAD, NADH, and the sodium salts of fructose-1,6-P_2 and fructose-1-P were purchased from Sigma Chemical Co. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), triose phosphate isomerase (EC 5.3.1.1) from rabbit muscle and a mixture of glycerolphosphate dehydrogenase (EC 1.1.1.8) and triose phosphate isomerase (rabbit muscle) were obtained as crystalline suspensions from Sigma Chemical Co. Nitroblue tetrazolium (Sigma Chemical Co.) phenazine methosulfate (Aldrich Chemical Co.), Amido Black 10B (Hartman-Leddon Co.), Noble agar (Difco Laboratories, Inc.) were commercial products. Acrylamide and *N,N'*-methylenebisacrylamide were products of Eastman Chemical Co. and were recrystallized according to the procedure of LOENING[12]. DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals, Inc. Urea and ammonium sulfate were the ultra-pure grade from Mann Research Laboratories. Other compounds used in these studies were commercial preparations of the highest grade available.

Animals

Adult male and gravid female frogs of *Rana pipiens* and *Rana catesbeiana* were purchased from The Lemberger Co., Oshkosh, Wisc. Gravid females of *R. pipiens* that had been induced to ovulate by the injection of a pituitary suspension[13] were obtained in this form from the commercial source. Eggs were stripped 24–48 h after injection

and were used immediately either for enzymatic studies or were fertilized with a sperm suspension according to the method of RUGH[13]. In the latter case, the fertilized eggs were placed in the medium described by BROWN AND CASTON[14] in large glass dishes in a water-bath maintained at 22° and allowed to develop. Embryos were staged according to the system described by SHUMWAY[15]. Under the conditions used, the first cell division was observed after 3 h. The stages of gastrulation, neural fold, muscular response and hatching were observed 1, 2, 3 and 4 days after fertilization, respectively.

Preparation of tissue extracts

The procedures used for the preparation of the extracts of adult frog tissues were those described previously[11]. In the studies on oogenesis and development, several eggs, or embryos at a given stage of development following fertilization, were used for the preparation of the tissue extracts. Jelly coats were removed manually and the embryos were homogenized with 50 μl of 0.06 M barbital buffer (pH 8.6) containing 2.5 mM β-mercaptoethanol and 1 mM EDTA. The homogenates were centrifuged at 30 000 $\times g$ for 10 min and the resulting supernatant solutions were used for enzymatic assay and for zone electrophoresis.

Standard assay for aldolase

Aldolase was determined spectrophotometrically by a modification of the method of BLOSTEIN AND RUTTER[16] as outlined earlier[11]. Protein concentrations were measured by the method of LOWRY *et al.*[17] with bovine serum albumin as the standard. In the case of the crystalline frog muscle aldolase, the protein concentration was determined spectrophotometrically, assuming an $E^{1\%}_{1cm} = 0.91$, the value calculated for rabbit muscle aldolase by BARANOWSKI AND NIEDERLAND[18].

Electrophoretic experiments

Unless otherwise stated, zone electrophoresis was performed in 0.04 M sodium barbital buffer (pH 8.5), containing 1 mM EDTA and 2.5 mM β-mercaptoethanol, on cellulose acetate strips (Gelman Sepraphore III, 2.5 cm $\times$ 17 cm) at 25 V/cm for 2 h at 4°. The strips were stained for aldolase activity as described previously[11]. Disc gel electrophoresis was carried out at pH 8.9 by the method of ORNSTEIN AND DAVIS[19,20].

Preparation of antisera to frog muscle aldolase

The procedure used for the isolation of aldolase from frog muscle (*R. catesbeiana*) was based on the one described by TAYLOR, GREEN AND CORI[21] for the crystallization of the rabbit muscle enzyme and will be described elsewhere[31]. The enzyme, which was recrystallized three times, gave a single protein band when subjected to polyacrylamide disc gel electrophoresis at different protein concentrations. In sedimentation velocity studies the protein sedimented as a single symmetrical peak. Prior to its use as an antigen, the enzyme was dissolved in 0.15 M NaCl and dialyzed against the same solution until free of ammonium ions.

Rabbits were immunized by weekly subcutaneous injections with 2 ml of an emulsion prepared from equal volumes of enzyme (10 mg/ml) and complete Freund's adjuvant. At the end of a 6-week period, all animals exhibited an antibody titer of at least 1:100 000 as monitored by the interfacial ring test[22]. Animals were bled by

cardiac puncture and given antigen booster injections (3 mg of enzyme in complete Freund's adjuvant) as necessary to maintain the antibody titer at the above level. The antisera and normal sera from noninjected rabbits were partially purified by sodium sulfate fractionation[23].

Agar gel double diffusion experiments

Double diffusion tests[24] were performed in 0.9% agar gel containing 2% NaCl and 5 mM EDTA (pH 7.5). Diffusion was allowed to proceed for 24–48 h at room temperature. Protein was stained with 0.5% Amido Black in 1% acetic acid for 5 min and then destained with a mixture of methanol–water–acetic acid (50:50:10, by vol.).

General procedures used in enzyme fractionation and chromatography

All operations were carried out at 4°. The ammonium sulfate solution, which was saturated at room temperature, contained 1 mM EDTA and was adjusted to pH 7.6 with ammonium hydroxide. Fractionations were carried out by the slow addition, with stirring, of the calculated amount of the saturated solution. The resulting suspensions were equilibrated for 30 min prior to centrifugation.

The DEAE-Sephadex A-50 ion exchanger used in column chromatography was equilibrated with the appropriate buffer before use. Columns were poured in the cold and then washed with several bed volumes of buffer.

RESULTS

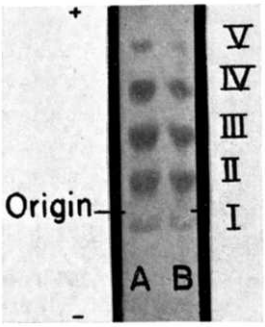
Zone electrophoresis of tissue extracts

The isozyme patterns of aldolase activity obtained when extracts of ovulated eggs and of egg + ovary preparations were subjected to electrophoresis and enzymatic staining are shown in Fig. 1. Both preparations show five bands of aldolase activity. The same electrophoretic pattern was observed when a single frog egg was removed from the ovary manually and the extract subjected to zone electrophoresis. On the basis of electrophoretic mobility, these isozymes have been numbered I through V, consecutively, I being localized at the most cathodic site and V at the most anodic site (see Fig. 1).

The electrophoretic patterns of aldolase isozymes in extracts of adult frog tissues were compared to those from egg preparations. Muscle and spleen extracts show only one band of aldolase activity which corresponds in electrophoretic mobility to that of isozyme I of egg. Liver has two bands of activity, one of which corresponds with that of muscle and a second, more anodic, band which is specific for liver. Five isozymes are present in extracts of heart, testes and brain and the electrophoretic patterns are similar to those observed with the egg preparations (see Fig. 1). The major activity band of brain coincides with that of isozyme V of egg.

Separation of aldolase isozymes in extracts of egg + ovary by chromatography on DEAE-Sephadex A-50

Although the results given above are consistent with the fact that the isozymes of aldolase found in the unfertilized egg are of the A–C types, more definitive studies were required to establish this fact. This necessitated the separation of the isozymes so that their individual properties could be investigated.



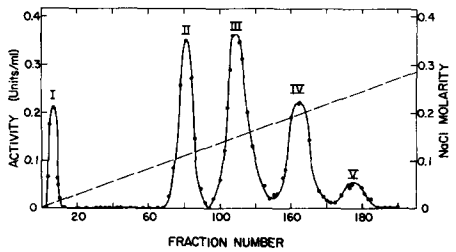


Fig. 1. Electrophoretic patterns of aldolase activities in extracts of egg + ovary (A) and of ovulated egg (B) from *R. pipiens*, Conditions used for the preparation of tissue extracts, electrophoresis and activity staining are described under METHODS. For reference purposes, Roman numerals, I through V, have been assigned to the isozymes on the basis of their relative electrophoretic mobilities.

Fig. 2. Separation of aldolase isozymes in extracts of egg + ovary by chromatography on DEAE-Sephadex A-50. The preparation of the extract and chromatographic procedures are described in the text. Fraction size = 5 ml. Aldolase activity (●—●) was determined by the standard assay system described under METHODS. Fractions with major aldolase activity of each individual peak were pooled and concentrated by ultrafiltration. — — —, molarity of NaCl.

Preliminary experiments established that a better separation of the isozymes could be achieved by chromatography of extracts from egg + ovary than with those from ovulated eggs. Hence, since the electrophoretic patterns of the isozymes from the two sources were identical (see Fig. 1), egg + ovary from gravid females of *R. pipiens* was used. The tissue was homogenized in 2 vol. (w/v) of 0.01 M Tris–HCl (pH 7.5) containing 1 mM EDTA and 10 mM β-mercaptoethanol. The homogenate was centrifuged at 35 000 $\times$ *g* for 30 min. Aldolase activity in the supernatant solution was recovered by ammonium sulfate fractionation (54–86% of saturation). The precipitate was dissolved in 0.05 M Tris–HCl buffer (pH 7.5) containing 1 mM EDTA and 10 mM β-mercaptoethanol. The resulting solution, which was dialyzed against the same buffer until free of ammonium ions, was applied to a column of DEAE-Sephadex A-50 (2.5 cm $\times$ 30 cm). The column was eluted with a linear gradient of NaCl (0–0.4 M; a total gradient volume of 1650 ml). The elution profile of aldolase

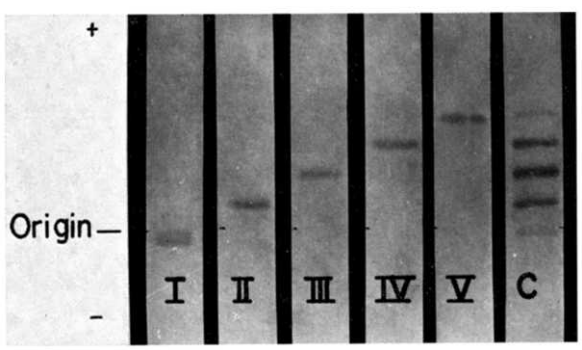


Fig. 3. Zone electrophoresis of resolved aldolase isozymes from egg + ovary. C = crude extract of egg + ovary; I through V = pooled concentrated fractions of isozymes separated by chromatography on DEAE-Sephadex A-50 (see Fig. 2). Zone electrophoresis and activity staining were carried out as described under METHODS.

activities is shown in Fig. 2. The distribution of activity of the five isozymes in Peaks I–V was 7.4%, 27%, 43%, 21% and 1.5%, respectively. The fractions containing aldolase activity and corresponding to the major portion of each individual peak were pooled and concentrated to one-tenth of the pooled volume by ultrafiltration (Diaflo apparatus, Amicon Inc., Cambridge, Mass.). The resolution of the isozymes by this procedure was confirmed by zone electrophoresis on cellulose polyacetate strips (Fig. 3). On the basis of electrophoretic mobilities, the isozymes from Peaks I and V correspond to muscle aldolase (type A) and brain aldolase (type C), respectively; the isozymes from Peaks II–IV are presumed to be the hybrids of these two parental types, *i.e.* A_3C, A_2C_2, and AC_3. Further evidence relating to this point is presented below. These fractions were the source of the resolved frog egg isozymes used in subsequent studies.

Separation of aldolase isozymes from extracts of frog brain by chromatography on DEAE-Sephadex A-50

A separation of frog brain isozymes was required to permit a comparison of their properties with those of the isozymes from egg. Brains from forty frogs *(R. pipiens)* were homogenized in 2 vol. (w/v) of 0.05 M Tris–HCl buffer (pH 7.5), containing 1 mM EDTA and 10 mM β-mercaptoethanol. The homogenate was centrifuged at 100 000 $\times g$ for 40 min. The resulting supernatant solution was fractionated with ammonium sulfate and the residue (35–65%) was recovered. The precipitate was dissolved in a minimum volume of the homogenizing buffer. The resulting solution (20 mg of protein), which was dialyzed against the same buffer until free of ammonium ions, was applied to a column (2 cm $\times$ 25 cm) of DEAE-Sephadex A-50. The isozymes were eluted with a linear gradient of NaCl (0–0.2 M; a total gradient volume of 800 ml). The elution pattern of aldolase activities obtained under these conditions is shown in Fig. 4. The distribution of activity of the five isozymes in Peaks I–V was 1.4%, 6.1%, 19.6%, 42.8% and 30.1%, respectively. The fractions with major aldolase activity of each peak were pooled and concentrated to one-tenth the pooled volume by ultrafiltration. A single band of aldolase activity was observed with each of the fractions upon zone electrophoresis (Fig. 5). These fractions were used in the subsequent studies with the individual isozymes from brain.

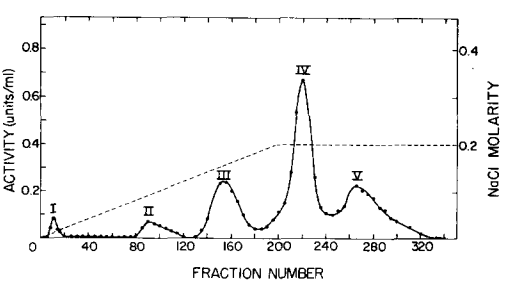


Fig. 4. Separation of aldolase isozymes in extracts of frog brain by chromatography on DEAE-Sephadex A-50. The preparation of the extract and chromatographic procedures are described in the text. Other conditions are those described in the legend to Fig. 1. Fractions with major aldolase activity of each individual peak were pooled and concentrated by ultrafiltration.

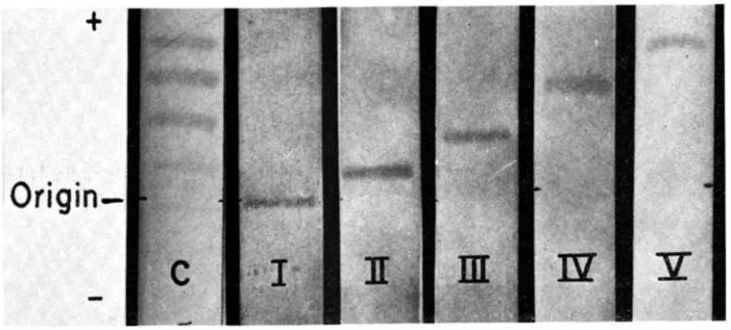


Fig. 5. Zone electrophoresis of resolved aldolase isozymes from frog brain. C = crude extract of brain; I through V = pooled concentrated fractions of isozymes separated by chromatography on DEAE-Sephadex A-50 (see Fig. 4). Electrophoresis and activity staining were carried out as described under METHODS.

Agar gel double diffusion studies

Double diffusion antigen–antibody reactions were carried out with the resolved isozymes by the OUCHTERLONY[24] technique. The results obtained when antibody against muscle aldolase (anti-A_4) was allowed to react with isozyme I from both egg + ovary and brain and with a crude muscle extract from *R. pipiens* are shown in Fig. 6. A continuous precipitin line, which fused completely, was formed indicating that isozyme I from either source is immunologically identical to muscle type aldolase. Similar results were obtained with isozyme I isolated from ovulated eggs.

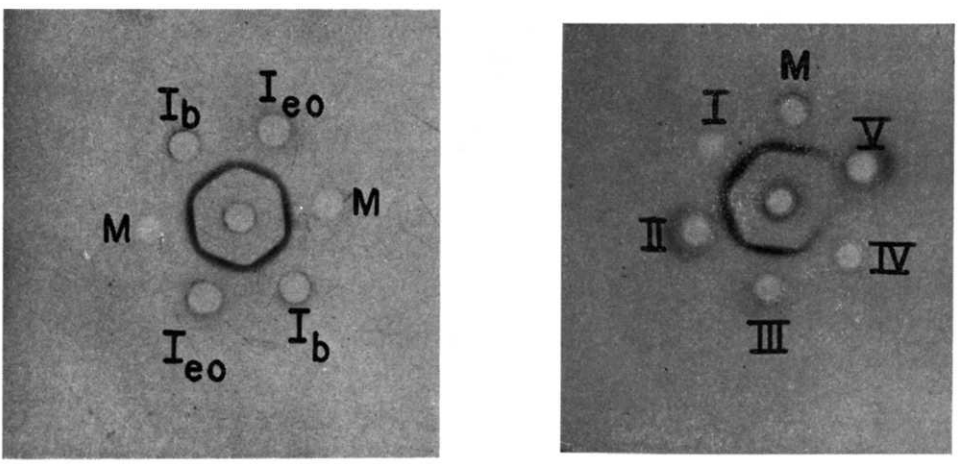
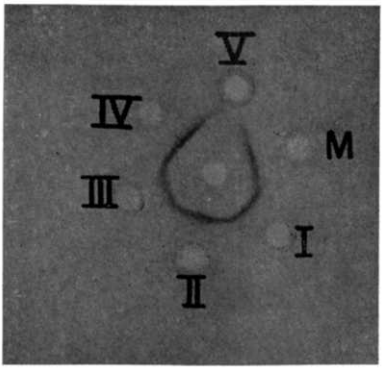


Fig. 6. Agar gel double diffusion experiments with muscle aldolase and isozymes I from brain and from egg + ovary. The antibody against the crystalline frog muscle aldolase was placed in the center well. Peripheral wells contained: I_b = isozyme I from brain separated by chromatography on DEAE-Sephadex A-50; I_{eo} = isozyme I from egg + ovary separated by the same procedure; and M = crude muscle extract (*R. pipiens*). Experimental conditions are described under METHODS.

Fig. 7. Agar gel double diffusion experiments with resolved isozymes from frog egg + ovary. The center well contained the antibody against crystalline frog muscle aldolase. Peripheral wells contained: M = crude muscle extract (*R. pipiens*); and I through V = isozymes I through V from egg + ovary separated by chromatography on DEAE-Sephadex A-50. Experimental conditions are described under METHODS.



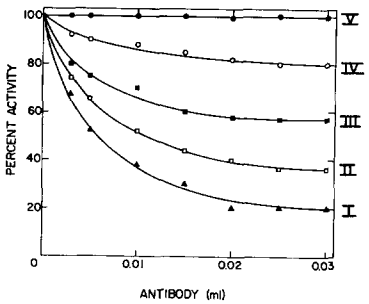


Fig. 8. Agar gel double diffusion experiments with resolved isozymes from frog brain. The center well contained the antibody against crystalline frog muscle aldolase. Peripheral wells = isozymes I through V from frog brain separated by chromatography on DEAE-Sephadex A-50. Experimental conditions are described under METHODS.

Fig. 9. Effect of the antibody to frog muscle aldolase on the activities of the resolved aldolase isozymes from frog egg + ovary. The same amount of each isozyme, based on units of activity (36 units) was mixed with increasing volumes of antibody. The antibody, enzyme, and other components of the standard assay system described under METHODS were preincubated for 1 min at room temperature before the addition of substrate (fructose-1,6-P_2). Partially purified serum from nonimmunized rabbits was used in place of the antibody in control experiments. I through V = resolved isozymes from egg + ovary.

Double diffusion experiments, in which anti-A_4 was allowed to react with the resolved isozymes from both egg + ovary and from brain, are shown in Figs. 7 and 8. Isozymes I through IV from both tissues formed a continuous precipitin line which fused completely with that of the crude muscle extract. There was no detectable cross-reaction with isozyme V from either brain or egg + ovary. These results are consistent with the fact that A subunits form a part, or all, of the structures of isozymes I through IV from both sources.

Inhibition of activity of resolved isozymes by antibody to aldolase A

The effects of the antibody against muscle aldolase on the activity of the resolved isozymes from egg + ovary (Fig. 9) and from brain (Fig. 10) were investigated. Anti-A_4 almost completely inhibited the activity of isozyme I from both brain and egg +

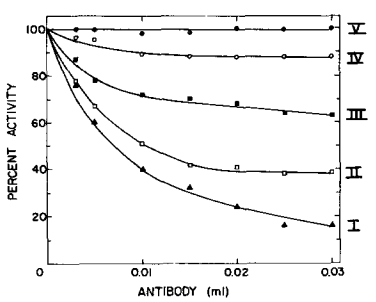


Fig. 10. Effect of antibody to crystalline frog muscle aldolase on the activities of the resolved isozymes from frog brain. Experimental conditions were those described in the legend to Fig. 9. I through V = resolved isozymes from frog brain.

TABLE I

SUBSTRATE SPECIFICITY OF RESOLVED ALDOLASE ISOZYMES FROM EGG + OVARY AND FROM BRAIN

Isozymes were separated by chromatography on DEAE-Sephadex (see Figs. 2 and 4). Assay conditions and other experimental details are described under METHODS.

Tissue	*K_m for fructose-1-P ($M \times 10^2$)*	*Fructose-1,6-P_2/fructose-1-P activity ratio*
Egg + ovary		
Isozyme V	1.6	15.1
IV	1.1	10.5
III	0.7	8.8
II	1.0	7.2
I	0.9	6.0
Brain		
Isozyme V	1.1	14.0
IV	0.8	9.5
III	0.9	8.0
II	1.2	6.5
I	0.8	5.5

ovary and had a decreasing effect on isozymes II–IV (II > III > IV). No inhibition of isozyme V from either tissue was observed. The inhibition of aldolase activity in crude muscle extracts was the same as that observed for isozyme I.

Substrate specificity of resolved isozymes

The substrate specificity of the resolved isozymes from egg + ovary and from brain was investigated (Table I). The apparent K_m values for fructose-1-*P* were determined from replots of initial velocity–substrate curves as outlined earlier for tissue extracts[11]. The fructose-1,6-P_2/fructose-1-*P* activity ratios have been calculated on the basis of the apparent V_{max} for fructose-1-*P*. The activity ratios of the resolved isozymes from both tissues fall in an ordered series between those of the parental types (A and C). The corresponding isozymes separated from egg + ovary and from brain have similar values indicating, therefore, that these two tissues have similar types of aldolase. In addition, the apparent K_m for fructose-1-*P* for all of the isozymes are of the same order of magnitude.

Formation of hybrid molecules of aldolase in vitro

If the isozymes of aldolase which are found in the egg are tetramers containing subunits of types A and C, dissociation–reassociation of a single hybrid form should led to the random recombination of the subunits with the generation of the other four forms of aldolase[3,9,25]. To investigate this point, isozyme III isolated from egg + ovary was subjected to such treatment[3]. Zone electrophoresis before and after reversible dissociation demonstrated that all five members of the A–C set were produced from a single isozyme by this procedure (Fig. 11).

Electrophoretic studies on aldolase isozymes during oogenesis and development

Possible changes in the electrophoretic patterns of aldolase isozymes during

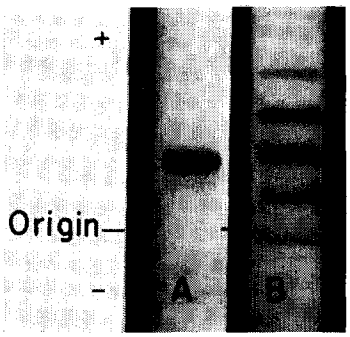


Fig. 11. Dissociation–reassociation of aldolase isozyme III isolated from egg + ovary. Isozyme III, which was isolated from egg + ovary by chromatography on DEAE-Sephadex A-50 (see Fig. 2) was subjected to dissociation and reassociation as described by PENHOET *et al.*[3]. A = before and B = after dissociation–reassociation. Experimental conditions used for zone electrophoresis and enzymic staining are those described under METHODS.

oogenesis were investigated. Eggs of three different sizes (approximate diameter = 0.3, 0.5 and 1.4 mm) were removed manually from ovaries and extracts prepared. The three preparations had essentially the same electrophoretic patterns, *i.e.* five aldolase isozymes of the A–C set.

The electrophoretic patterns of aldolase isozymes at different developmental stages were investigated. The methodology used in these studies is described under METHODS. The isozyme pattern of aldolases in the unfertilized egg was compared to that of embryos at the following stages[15]: 11 (midgastrula); 14 (neural fold); 15 (rotation); 17 (tail bud); 18 (muscular response); 20 (hatching); and 25 (operculum complete). There was essentially no change in the isozyme pattern from the unfertilized egg up to the stage of muscular response; the same A–C hybrid set was observed and no qualitative changes in the relative intensities of the isozymes was noted. At muscular response, there was a marked increase in aldolase A_4. Liver aldolase (type B) was not detected until 2 days after hatching.

DISCUSSION

A primary purpose of this work was to study the types of aldolase isozymes present in various amphibian tissues at different stages of development with particular reference to those found in the unfertilized egg. The results, which confirm earlier electrophoretic studies[11], clearly establish that the unfertilized frog egg contains aldolases A and C together with their hybrids. This conclusion is based on the fact that the individual isozymes from both egg and brain had similar electrophoretic mobilities and substrate specificities which were significantly different from those established previously for frog liver aldolase (type B)[11]. Furthermore, immunological studies with the antibody to muscle aldolase demonstrated that the activity of isozyme I (A_4) from either egg or brain, as well as from muscle, was almost completely inhibited whereas no inhibition was observed with isozyme V (C_4); progressively decreasing inhibition was observed with isozymes I–IV from either source. In agar gel double diffusion experiments, anti-A_4 was found to react with isozymes I–IV but not with isozyme V. These findings show that A and C type frog aldolases are anti-

genically different and are, therefore, presumably controlled by independent structural genes as suggested by PENHOET *et al.*[25] and MASTERS[9]. The fact that the dissociation–reassociation *in vitro* of isozyme III (A_2C_2) from egg led to the formation of the other four isozymes, as predicted for a tetramer with two different types of subunits, ruled out the possibility that the isozymes found in the egg are formed from a single polypeptide chain by modification as discussed by MARKERT[26].

The presence of aldolases of both the A and C types in the unfertilized frog egg suggests that they are not functionally equivalent during development. On the basis of substrate specificities, tissue distribution, and other properties, RUTTER and associates[27,28] have suggested that muscle aldolase (type A) is primarily involved in glycolysis, whereas liver aldolase (type B) plays a role in gluconeogenesis and fructose metabolism. Since the first energy-rich compound to be catabolized in the developing frog embryo is glycogen (*cf.* ref. 29), the occurrence of aldolase A in the egg is consistent with the physiological role ascribed to this isozyme. To date, no specific physiological function has been suggested for C type aldolase. LEBHERZ AND RUTTER[2] have pointed out that the enzyme may have undiscovered catalytic properties, or a unique intracellular distribution which accounts for a specific function. Since many studies on aldolase isozymes have been carried out on tissues composed of a heterogeneous population of cells, it is not always clear whether the various cell types contribute different isozymes to the total forms observed. It has been shown that different parts of an organ have different types of isozymes. For example, aldolase A was the only activity found in the medulla of the rabbit kidney, while aldolases of the cortex consisted of the five membered A–B hybrid set[2]. On the other hand, in the present studies it was shown that a single frog egg has the five isozymes of the A–C set. Hence, both A and C type aldolases are present in a single cell. Individual isozymes of lactate dehydrogenase have been shown to have a specific intracellular distribution in the frog egg[30]. It remains to be established whether this is true for the aldolases.

ACKNOWLEDGMENTS

This study was supported in part by Research Contract No. AT(11-1)-1631 from the U.S. Atomic Energy Commission and by Grant No. AM-00922 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service. The technical assistance of Mr. EDWARD KMIOTEK in certain phases of this work is gratefully acknowledged.

REFERENCES

1 W. J. RUTTER, T. RAJKUMAR, E. PENHOET, M. KOCHMAN AND R. VALENTINE, *Ann. N.Y. Acad. Sci.*, 151 (1968) 102.
2 H. G. LEBHERZ AND W. J. RUTTER, *Biochemistry*, 8 (1969) 109.
3 E. PENHOET, T. RAJKUMAR AND W. J. RUTTER, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1275.
4 K. KAWAHARA AND C. TANFORD, *Biochemistry*, 5 (1966) 1578.
5 C. L. SIA AND B. L. HORECKER, *Arch. Biochem. Biophys.*, 123 (1968) 186.
6 F. J. CASTELLINO AND R. BARKER, *Biochemistry*, 7 (1968) 2207.
7 C. S. WEBER AND W. J. RUTTER, *Federation Proc.*, 23 (1964) 487.
8 V. RENSING, A. SCHMIDT AND F. LEUTHARDT, *Z. Physiol. Chem.*, 348 (1967) 921.
9 C. J. MASTERS, *Biochim. Biophys. Acta*, 167 (1968) 161.
10 J. J. HERSKOVITZ, C. J. MASTERS, P. M. WASSARMAN AND N. O. KAPLAN, *Biochem. Biophys. Res. Commun.*, 26 (1967) 24.

11 K. ADACHI, L. J. CHEN AND H. J. SALLACH, *Biochem. Biophys. Res. Commun.*, 30 (1968) 343.
12 U. E. LOENING, *Biochem. J.*, 102 (1967) 251.
13 R. RUGH, *Experimental Embryology: A Manual of Techniques and Procedures*, Burgess, Minneapolis, Minn., 1961, p. 102.
14 D. D. BROWN AND D. J. CASTON, *Develop. Biol.*, 5 (1962) 412.
15 W. SHUMWAY, *Anat. Record*, 78 (1940) 139.
16 R. BLOSTEIN AND W. J. RUTTER, *J. Biol. Chem.*, 238 (1963) 3280.
17 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
18 T. BARANOWSKI AND T. R. NIEDERLAND, *J. Biol. Chem.*, 180 (1949) 543.
19 L. ORNSTEIN AND B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 305.
20 L. ORNSTEIN AND B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 385.
21 J. F. TAYLOR, A. A. GREEN AND G. F. CORI, *J. Biol. Chem.*, 173 (1948) 591.
22 E. A. KABAT AND M. M. MAYER, *Exptl. Immunochem.*, Charles C. Thomas, Springfield, Ill., 1961, p. 72.
23 P. A. SMALL, JR., R. A. REISFELD AND S. DRAY, *J. Mol. Biol.*, 11 (1965) 713.
24 O. OUCHTERLONY, *Arkiv Kemi*, B26 (1949) 1.
25 E. PENHOET, M. KOCHMAN, R. VALENTINE AND W. J. RUTTER, *Biochemistry*, 6 (1967) 2940.
26 C. L. MARKERT, *Ann. N.Y. Acad. Sci.*, 151 (1968) 14.
27 W. J. RUTTER, R. BLOSTEIN, B. M. WOODFIN AND C. S. WEBER, *Symposium on Regulation of Enzyme Activity and Synthesis in Normal and Neoplastic Liver, Indianapolis, October, 1962*, Pergamon, London, 1963.
28 W. J. RUTTER, R. E. BLOSTEIN, B. M. WOODFIN AND C. S. WEBER, *Advan. Enzyme Reg.*, 1 (1963) 39.
29 G. W. BROWN, JR., in J. A. MOORE, *Physiology of the Amphibia*, Academic Press, New York, 1964, p. 1.
30 G. W. NACE, T. SUYAMA AND N. SMITH, *Symposium on Germ Cells and Development*, Inst. Internat. d'Embryol., Baselli, 1960, p. 564.
31 L. J. CHEN AND H. J. SALLACH, in preparation.