Stability of Quaternary Structure of Mammalian and Avian Fructose Diphosphate Aldolases†

Herbert G. Lebherz

ABSTRACT: Studies on isolated rabbit and chicken parental (A, B, C) and hybrid (A-C) fructose diphosphate (FDP) aldolases have shown that: (1) (a) the aldolases can be maintained at very low protein concentration without loss of activity; (b) inactivation of aldolase commonly observed at low concentration most likely results from inactivation of the enzymes on vessel surfaces; (c) the rates of inactivation of parental and hybrid aldolases increase as the number of positively charged subunits (A and B) in the tetramer increase; (d) glass surface inactivation of aldolase can be prevented or retarded, but not reversed, by low concentrations (1×10^{-5} to 1×10^{-4} M) of FDP; (2) the aldolase tetramer does not dissociate at enzyme concentrations as low as 0.0002 mg/ml in the presence or absence of FDP as judged by gel filtration and subunit exchange experiments; (3) unlike lactate dehydrogenase, (a) subunit exchange between parental aldolases (A and C) was not observed under *in vitro* conditions which did not result in reversible loss of enzymatic activity; (b) hybrids were not formed by freezing and thawing parental aldolases (A and C) in high salt concentrations; (c) at low pH, the quaternary structure of hybrid (AC_3) and parental (A_4) aldolases appear to be of comparable stability. From these observations, it is concluded that the tetramer is the catalytically active species of mammalian and avian FDP aldolases and that substrate is not required to maintain the tetrameric structure of these molecules under conditions of enzyme assay. The stability of aldolase quaternary structure is discussed in relation to the possible role subunit exchange between multiple forms of enzymes may play in controlling the levels of isoenzymes within the cell.

The three homologous FDP[1] aldolases A, B, and C isolated from mammalian tissues have molecular weights of approximately 160,000 and are composed of four subunits (Kawahara and Tanford, 1966; Penhoet *et al.*, 1967, 1969b). Homologs of the mammalian enzymes have been detected in a wide variety of vertebrates and these aldolases also appear to be tetramers (Lebherz and Rutter, 1969). In addition to the three parental aldolases, hybrid molecules formed by the combination of the parental subunits into tetramers can be produced *in vitro* by reversible acid dissociation (Penhoet *et al.*, 1966) and are observed in tissues in which two of the parental forms are synthesized (Lebherz and Rutter, 1969; Lebherz, 1972).

Of the vertebrate aldolases, the rabbit muscle enzyme has been the most extensively studied concerning the stability of tetrameric structure under denaturating and nondenaturating conditions. This enzyme can be dissociated to monomeric subunits by a variety of agents including hydrogen ions, urea, and sodium dodecyl sulfate (Stellwagen and Schachman, 1962; Deal *et al.*, 1963) or to dimers by less rigorous dissociating conditions (Blatti, 1968). Removal of the dissociating agents results in the reconstitution of tetramers which have catalytic, molecular, and immunological properties virtually identical with those of the native enzyme (Stellwagen and Schachman, 1962; Deal *et al.*, 1963). There have also been reports that aldolase A dissociates at low-enzyme concentration. Using sedimentation equilibrium ultracentrifugation, Kawahara and Tanford (1966) observed a decrease in the molecular weight of rabbit aldolase A at protein concentrations below 0.2 mg/ml and suggested that this decrease resulted from dissociation of the tetrameric species. Bernfeld *et al.* (1965) have interpreted the commonly observed inactivation of aldolase A at low concentration (less than 0.01 mg/ml) to be the result of reversible dissociation of active enzyme to inactive species.

There has yet to be a direct determination of the molecular species of FDP aldolase that prevails under conditions of enzyme assay. The above-mentioned data of Kawahara and Tanford (1966) suggest that dissociated species would predominate at protein concentrations used for activity measurements (on the order of 0.001 mg/ml). In contrast, Blatti (1968) has presented kinetic evidence that aldolase dimers produced by low pH must reassociate to tetramers before activity is observed and, therefore, that the tetramer is the active species. Finally, the report (Masters and Winzor, 1971) that dissociation of aldolase A normally seen in 1.5 M urea at pH 5 can be prevented by FDP and fructose 1-phosphate suggests that the tetramer is the active unit, and that the enzyme's quaternary structure is influenced by substrate.

The present studies on isolated rabbit and chicken FDP aldolases were undertaken: (1) to investigate the stability of aldolase activity at low-enzyme concentrations in the hope of determining whether inactivation at high dilution occurs as a result of enzyme dissociation, as suggested by Bernfeld *et al.* (1965), or as a result of other processes; (2) to determine directly the state of aggregation of the mammalian and avian enzymes under conditions of enzyme assay in order to establish the structure of catalytically active FDP aldolases; and (3) in view of the recent observations that subunit exchange between LDH isoenzymes occurs under mild *in vitro* conditions and the suggestions that subunit exchange may be involved in the control of the levels of these isoenzymes *in vivo* (Millar *et al.*, 1971; Fritz *et al.*, 1971), to test for subunit

† From the Laboratory for Developmental Biology, Zoology Institute. Swiss Federal Institute of Technology, Zürich, Switzerland. *Received February 4, 1972.* This research was supported by the Swiss National Science Foundation, Project No. 3.247.69.

[1] Abbreviations used are: FDP, fructose diphosphate; ME, 2-mercaptoethanol; LDH, lactate dehydrogenase; TDH, triosephosphate dehydrogenase.

exchange between aldolases, especially under conditions in which the enzymes remain totally active.

Materials

Rabbit skeletal muscle, liver, and brain were obtained from a local butcher. Frozen chicken skeletal muscle, heart, and brain were acquired through the courtesy of Micarnia SA, Courtepin, Switzerland.

All substrates, coenzymes, and enzymes other than FDP aldolases were purchased from either the Sigma Chemical Co., St. Louis, Mo., or Boehringer Mannheim Co., Mannheim, Germany. Phosphocellulose (0.66 mVal/g) was obtained from Schleicher und Schüll, Feldbach, Switzerland. DEAE-Sephadex A-50 and Sephadex G-150 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Materials for electrophoresis, including Sepraphore III cellulose polyacetate strips, were obtained from Gelman Instrument Co., Ann Arbor, Mich. All other chemicals were of reagent grade.

Methods

Isolation of Rabbit and Chicken FDP Aldolases. Rabbit aldolases A and B were purified from skeletal muscle and liver, respectively. Aldolase C and the A-C hybrids were isolated from rabbit brain. The purification methods used have previously been described by Penhoet *et al.* (1969a) and involve (1) tissue homogenization, (2) ammonium sulfate fractionation of the soluble proteins, and (3) substrate elution from phosphocellulose. Aldolases A and B were obtained in essentially pure form after the phosphocellulose step and were either crystallized or precipitated from 60% ammonium sulfate. These preparations were freed of the FDP introduced at the time of substrate elution by suspension in 70% ammonium sulfate followed by centrifugation. Aldolase C and the A-C hybrid enzymes were purified further and separated from one another by chromatography on DEAE-Sephadex A-50 (Penhoet *et al.*, 1969a). The corresponding chicken enzymes were isolated from skeletal muscle (aldolase A), heart (A-C hybrids), and brain (aldolase C) in a similar manner.

Enzyme Assays. Aldolase activity using 2.5 mM FDP as substrate was determined using the coupled spectrophotometric assay of Blostein and Rutter (1963) at 25° in either 0.1 M Tris-HCl (pH 7.6) or 0.1 M glycylglycine (pH 7.6); a unit of activity is expressed as the cleavage of 1 μmole of substrate/min per ml of enzyme solution. TDH activity was determined by the method of Allison and Kaplan (1964) at 25°; a unit of activity is expressed as micromoles of NAD reduced per minute milliliter of enzyme solution.

Protein Determinations. During enzyme purification, protein concentrations were estimated by measuring the absorbance at 280 and 260 mμ according to the method of Warburg and Christian (1942). Protein concentrations of purified rabbit aldolase preparations were determined by measuring the absorbance at 280 mμ using the published extinction coefficients ($E^{0.1\%}_{280m\mu,\,1cm}$) of 0.91, 0.89, and 0.88 for aldolases A, B, and C, respectively (Baranowski and Neiderland, 1949; Penhoet *et al.*, 1969b). Protein concentrations of chicken aldolases A and C were estimated using the extinction coefficients of the corresponding rabbit enzymes. TDH concentrations were measured assuming an extinction coefficient at 280 mμ of 1.0 (Kochman and Rutter, 1968).

Electrophoresis. Cellulose polyacetate electrophoresis was performed in 0.06 M sodium barbital–0.06% ME (pH 8.6) according to the method of Penhoet *et al.* (1966). Samples were applied to the strips and electrophoresis carried out at 250 V for 90 min. Afterward, the strips were stained for activity as previously described (Penhoet *et al.*, 1966) or for protein with 1% Amido-Schwarz 10B in 7% acetic acid.

Results

Homologous FDP Aldolases Isolated from Rabbit and Chicken Tissues. Cellulose polyacetate electrophoretic profiles of the eleven aldolases isolated are presented in Figure 1. Single bands of activity were detected in each preparation. In addition, electrophoresis followed by protein staining with Amido Schwarz indicated that the enzymes were nearly homogeneous. The purified parental rabbit aldolases A, B, and C had specific activities of 15, 1, and 6 μmoles of FDP cleaved per min per mg of protein, respectively. The corresponding chicken A and C enzymes had specific activities of 16 and 8 units per mg of protein, respectively. Hybrid molecules containing both A and C subunits had specific activities intermediate between those of the A and C homotetramers. No attempt was made to isolate hybrid enzymes containing A and B subunits from either organism, nor was aldolase B purified from chicken liver.

Inactivation of Aldolase Activity at Low-Enzyme Concentration. Bernfeld *et al.* (1965) have interpretated the inactivation of aldolase A at protein concentrations below approximately 10 μg/ml to result from reversible dissociation of active enzyme into inactive components and suggested that, under these conditions, certain polycations and proteins protect aldolase activity by inhibiting dissociation of the active species. However, no attempt was made in their studies to reactivate the dilute enzyme by reconcentration. Reactivation by concentrating would be expected if, in fact, the aldolase molecule existed in equilibrium between associated (active) and dissociated (inactive) species at low protein concentration. The following studies on dilute aldolase solutions were performed in order to better understand the processes involved in the inactivation of aldolase at high dilution.

VESSEL SURFACE INACTIVATION OF ALDOLASE A. Inactivation of both rabbit and chicken aldolase A was observed when the enzymes were maintained in small volumes at concentrations below 10 μg/ml. Inactivation was especially prominent when incubations were performed in glass test tubes but was also observed in siliconized glass and nitrocellulose tubes. The time-dependent inactivation of chicken aldolase A at 1 μg/ml in glass tubes is shown in Figure 2. Inactivation was prevented by inclusion of 1 mg/ml of bovine serum albumin but inactivation was not reversed by subsequent addition of this protein. In contrast, no inactivation was observed for extended periods of time when the diluted enzymes were maintained in large volumes and in polypropylene plastic beakers. Even in glass beakers, little loss of activity was observed for several hours if large volumes of dilute enzyme (low surface area:volume ratio) were used. Preincubation of glass tubes with 10 μg/ml of aldolase A for 18 hr prior to carrying out incubations with 1 μg/ml of enzyme considerably retarded the inactivation normally seen in glass. It was also observed that the rate of inactivation of aldolase A could be increased by frequent swirling of the enzymes in glass test tubes or by transferring the enzyme solutions periodically to new tubes. These observations demonstrate that dilution alone does not cause inactivation of aldolase A; rather, they suggest that the inactivation commonly ob-

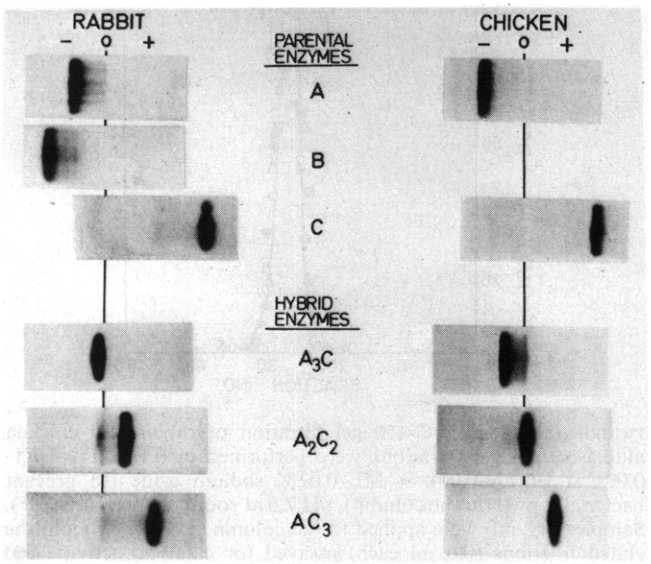

FIGURE 1: Cellulose polyacetate electrophoretic profiles of purified rabbit and chicken FDP aldolases. See Methods section for details on electrophoresis and staining for aldolase activity.

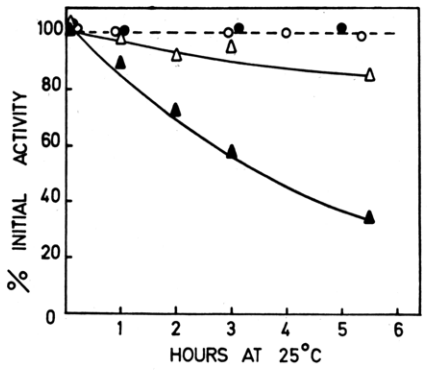

FIGURE 2: Inactivation of chicken aldolase A at low-enzyme concentration. Chicken aldolase A at 0.001 mg/ml was incubated at 25° in 0.01 M Tris-HCl–0.001 M EDTA–0.001 M dithiothreitol (pH 7.6). (▲) Glass test tube; (●) glass test tube containing 1 mg/ml of bovine serum albumin; (△) glass test tube preincubated for 18 hr at 25° with 0.01 mg/ml of aldolase A (final volume in each tube was 10.0 ml); (○) 50 ml of aldolase A (0.001 mg/ml) incubated in a 100-ml polypropylene plastic beaker. At the times indicated, 0.05-ml aliquots were removed for assay of FDP cleavage activity.

served at low enzyme concentration results from interactions between aldolase molecules and vessel surfaces.

ALDOLASE SUBUNIT COMPOSITION AND SURFACE INACTIVATION. The rates of inactivation of rabbit and chicken aldolases A were found to increase as the ratio of glass surface area to volume of enzyme was increased. Therefore, the aldolases were incubated with constant stirring in the presence of glass beads (washed in acid and EDTA), so that inactivation could be observed over a convenient time period. As shown in Figure 3, the relative rates of inactivation of the rabbit and chicken aldolases were dependent on the subunit compositions of the various tetramers. Rabbit and chicken aldolases C remained totally active for longer than 4 hours while the corresponding A enzymes lost 50% or more of their initial activities within 1 hr. Of the enzymes tested, rabbit aldolase B exhibited the highest rate of inactivation. Hybrid molecules containing both A and C subunits were inactivated at rates intermediate between those of the parental homotetramers; notice rabbit A_2C_2 and chicken A_3C. In the experiment presented in Figure 3, the chicken hybrid AC_3 was not inactivated by glass beads; however, in another experiment, some inactivation of this enzyme was observed. The rate of inactivation of chicken A_2C_2 (not shown) was intermediate between the other two chicken hybrids. Semilog plots of the data from Figure 3 showed that glass surface inactivation of the aldolases followed first-order kinetics.

SUBSTRATE PROTECTION AGAINST SURFACE INACTIVATION. Glass surface inactivation of the aldolases was retarded or prevented, but not reversed, by low concentrations of the substrate FDP. Substrate protection of aldolase A is illustrated in Figure 4. FDP at an initial concentration of 1×10^{-4} M afforded essentially complete protection against inactivation of the rabbit and chicken enzymes for up to 2 hr, while in the absence of substrate these aldolases were 80% inactivated during the same time. In addition, essentially no inactivation was observed for longer than 2 hr when chicken aldolase A (5 μg/ml) was maintained in glass tubes in the absence of glass beads and in the presence of 1×10^{-5} M FDP, while 30% loss of activity occurred within 2 hr in the absence of substrate. Finally, partial protection of aldolase B was observed with 8×10^{-6} M FDP. Using the data of Herbert *et al.* (1940), the equilibrium constant of the FDP $\rightleftharpoons$ 2-triose phosphate conversion at 22° was estimated to be ap-

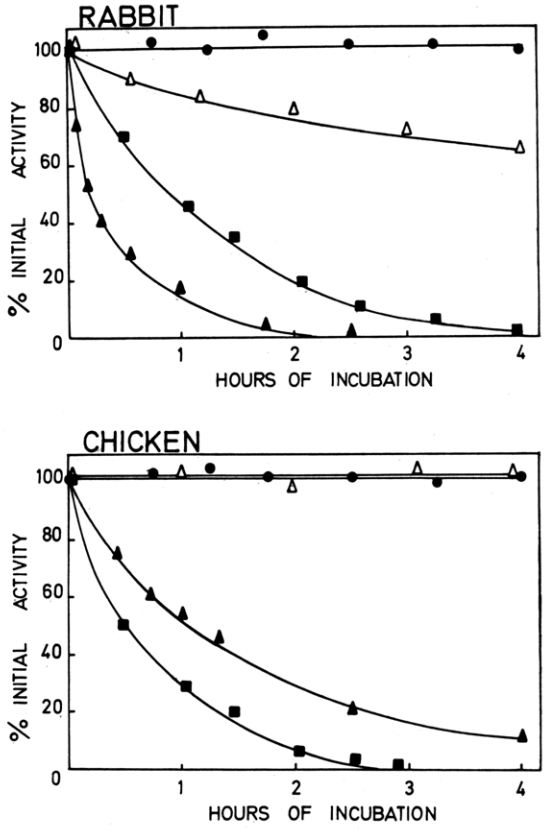

FIGURE 3: Effect of subunit composition on the inactivation of FDP aldolases by glass. The rabbit and chicken aldolases were incubated at 0.005 mg/ml in 0.1 M Tris-HCl–0.001 M EDTA–0.001 M ME (pH 7.6) in glass beakers containing 0.3 g/ml of acid and EDTA-washed glass beads (0.17–0.18 mm diameter) (final volume 30 ml). Rabbit: A_4 (■), B_4 (▲), C_4 (●), A_2C_2 (△); chicken: A_4 (■), C_4 (●), A_3C (▲), AC_3 (△). The solutions were incubated at room temperature (22°) with constant stirring and 0.05-ml aliquots removed at the times indicated for assay.

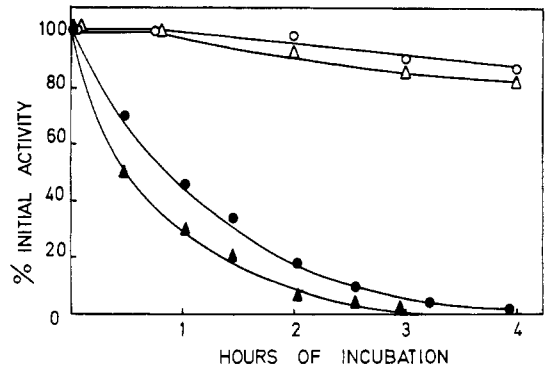

FIGURE 4: Effect of FDP on the inactivation of aldolase activity by glass beads. Rabbit (○) and chicken (△) aldolases A were incubated under the conditions described in the legend of Figure 3. Open symbols: $+1 \times 10^{-4}$ M FDP; closed symbols: −FDP. At the times indicated, 0.05-ml aliquots were removed for assay. Samples were assayed in the presence and absence of added FDP to correct for the presence of triose phosphates produced in the incubated mixtures containing FDP *via* the aldolase reaction since these components are substrates in the coupled aldolase assay.

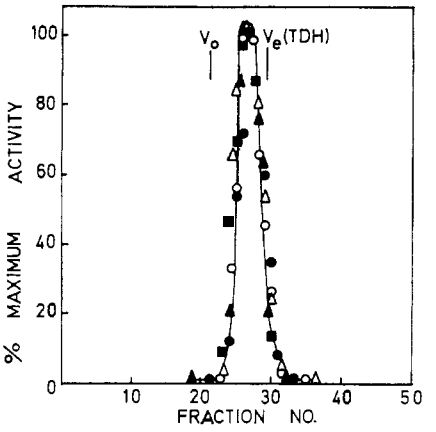

FIGURE 5: Sephadex G-150 gel filtration of rabbit and chicken aldolases. All gel filtrations were performed in 0.1 M Tris-HCl–0.001 M EDTA–0.001 M ME–0.02% sodium azide (to prevent bacterial growth in the column), pH 7.6 at room temperature (22°). Samples (0.2 ml) were applied to the column (1 × 54 cm) and the eluted fractions (0.6 ml each) assayed for catalytic activity. (●) Chicken aldolase C (4.0 mg/ml); (○) chicken aldolase C (0.004 mg/ml); (■) rabbit aldolase C (0.005 mg/ml); (▲) chicken aldolase A (0.005 mg/ml); (△) rabbit aldolase A (0.005 mg/ml). Aldolases A of both organisms were subjected to gel filtration in the presence of 1×10^{-4} M FDP. The elution volume of TDH, V_e (TDH), was determined with 0.2 ml of 6-mg/ml rabbit muscle TDH and the void volume, V_0, was determined with 0.1% Blue Dextran.

proximately 4×10^{-5} M. Therefore, with the low levels of substrate used here, the concentrations of FDP present at equilibrium (1×10^{-6} to 5×10^{-5} M) were somewhat lower than those added initially (8×10^{-6} to 1×10^{-4} M). It is interesting to note that the Michaelis constants for FDP of the rabbit and chicken aldolases A, B, and C have been reported to be between 1×10^{-6} and 7×10^{-5} M (Penhoet *et al.*, 1969b; Marquardt, 1969–1971). Therefore, the present observations suggest that FDP protection of the aldolases involves binding of substrate to the enzyme's active center.

Structure of Catalytically Active FDP Aldolase. As demonstrated above, the activity of rabbit and chicken aldolases can be maintained at high dilution if vessel surface inactivation is avoided. In this section, experiments are presented which show that these enzymes also retain their tetrameric structure at low concentration and that substrate is not required to maintain this structure.

GEL FILTRATION. Rabbit and chicken aldolases A and C at low concentration were subjected to Sephadex G-150 gel filtration. All experiments were performed at room temperature (22°) and in buffers normally used to measure catalytic activity. As shown in Figure 5, the elution profile of chicken aldolase C preincubated at 0.004 mg/ml for 8 hr was identical with that of a 1000-fold more concentrated sample (4 mg/ml) of the same enzyme. It is known that at 4 mg/ml, aldolase C exists as a tetramer (Penhoet *et al.*, 1969b). The profile of rabbit aldolase C at an initial concentration of 0.005 mg/ml was the same as those of the corresponding chicken enzyme. It was necessary to perform gel filtrations of dilute aldolase A solutions in the presence of FDP (1×10^{-4} M) since extensive loss of activity of these dilute enzyme solutions, presumably due to surface inactivation (see above), occurred in the absence of substrate. Under these conditions, the elution profiles of rabbit and chicken aldolases A at initial concentrations of 0.005 mg/ml were indistinguishable from those of the C enzymes. All of the enzymes tested were eluted in a single, symmetrical peak with greater than 80% recovery of activity and with an apparent molecular size greater than that of the marker enzyme TDH (V_e TDH). Although TDH may dissociate to dimers at enzyme concentrations below 0.5 mg/ml (Hoagland and Teller, 1969), the high concentration used for these gel filtrations (6 mg/ml) assured elution of this enzyme as a tetramer of approximately 140,000 (Harrington and Karr, 1965).

SUBUNIT EXCHANGE. Although the gel filtration experiments show that the rabbit and chicken aldolases behave as tetramers at low-enzyme concentration, they do not rule out the possibility that some tendency for the tetramers to dissociate may exist at high dilution. This possibility was tested by subunit exchange experiments. Aldolases A and C were maintained together and each A-C hybrid separately at enzyme concentrations of 0.001 mg/ml for 2 days at 25°. Afterward, the enzyme solutions were concentrated to approximately 0.15 mg/ml by ultrafiltration and subjected to electrophoresis (see legend of Figure 6 for details). Loss of activity after 2 days was always less than 20% and was usually much less (Figure 6). Whenever inactivation was observed, the lost activity could not be recovered by reconcentration. As shown in Figure 6, no subunit exchange between aldolase tetramers was apparent even after 2 days; A-C hybrids were not produced in solutions containing the parental A and C enzymes, nor were "new" members of the A-C set generated in solutions containing single rabbit or chicken A-C hybrid molecules. Similar results were obtained when 0.1 M glycylglycine (pH 7.5) was substituted for 0.1 M Tris-HCl, in the incubation buffers. Furthermore, no subunit exchange was observed after 2 days in solutions containing 0.0002 mg/ml of chicken A_3C or AC_3 (final volume = 800 ml). Finally, no A-B hybrids were evident after 1 day in solutions containing rabbit aldolases A and B.

The argument may be raised that the failure to detect subunit exchange between the parental aldolases or between tetramers of the symmetrical hybrid A_2C_2 does not rule out dissociation of these enzymes at low concentration. If dissociation of the parental enzymes to the dimers A_2 and C_2 had occurred, and if the dimer-dimer interactions between A_2–A_2 and/or between C_2–C_2 were very much greater than those between A_2–C_2, then only the parental tetramers would

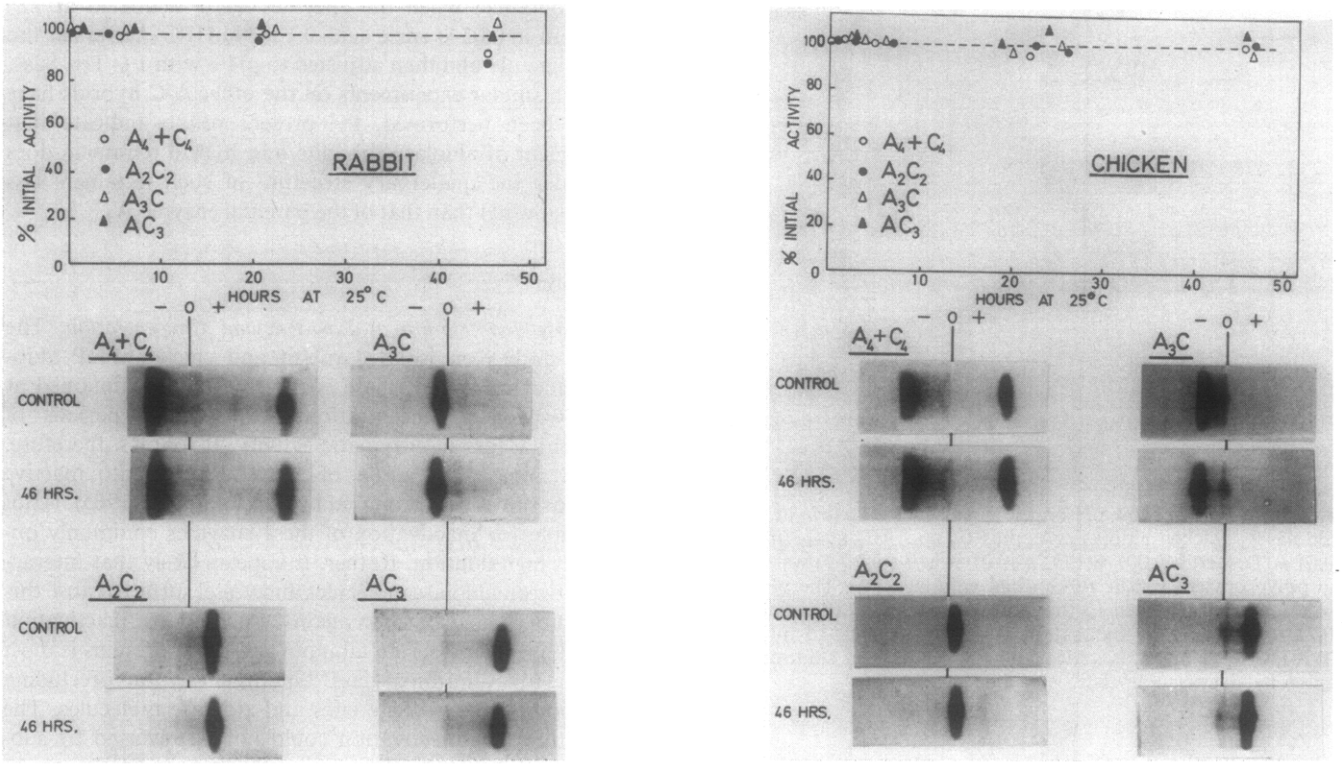

FIGURE 6: Effect of dilution on the activity and quaternary structure of rabbit and chicken FDP aldolases. The enzymes (0.001 mg/ml) were incubated in 0.1 M Tris-HCl–0.001 M EDTA–0.001 M ME (pH 7.6) at 25° in polypropylene plastic beakers (final volume 150 ml). At the times indicated, 0.05-ml aliquots were removed for assay. After 46 hr, the solutions were concentrated to approximately 0.15 mg/ml by passage through Diaflo PM-30 ultrafiltration membranes and subjected to electrophoresis.

be observed after reconcentration. Also, if the symmetrical hybrid A_2C_2 preferentially dissociated to produce only AC dimers then these dimers would, upon reconcentration, reassociate to form only the original A_2C_2 tetramer. But, no such arguments can be made for the results with the asymmetrical hybrids A_3C and AC_3. Surely, had dissociation of these tetramers occurred, "new" members of the A-C set would have formed upon reconcentration.

From these subunit exchange experiments, it is apparent that the rabbit and chicken aldolases do not dissociate at low-enzyme concentration even in the absence of substrate.

Further Studies on the Stability of Aldolase Tetrameric Structure. ATTEMPTS AT SUBUNIT EXCHANGE UNDER NONDENATURATING CONDITIONS. The recent reports that subunit exchange between LDH isoenzymes (Millar *et al.*, 1971; Fritz *et al.*, 1971) and between homologous forms of TDH (Spotorno and Hollaway, 1970) can be observed under conditions which do not require reversible enzyme denaturation prompted similar studies on the FDP aldolases. Advantage was taken of the observations that subunit exchange between the H and M forms of mammalian LDH is dependent on temperature, pH, and the presence of sulfhydryl reagent (Millar *et al.*, 1971). The present experiments with chicken aldolases A and C were performed in polypropylene plastic tubes at 40° in the presence of 0.005 M dithiothreitol and in a variety of buffer systems; namely, 0.1 M sodium phosphate (pH 5.9), 0.25 M sodium malonate (pH 6.2) ± 0.4 M NaCl, 0.05 M Tris-HCl (pH 7.5) ± 0.4 M NaCl, 0.1 M glycylglycine (pH 7.6), 0.02 M sodium phosphate–0.1 M NaCl (pH 7.9), and 0.1 M glycine (pH 9.0). The final protein concentration in each case was 0.2 mg/ml. Under all conditions employed, greater than 80% recovery of activity was observed after 1 day. In no instance was evidence of subunit exchange (hybrid formation) found even after 2-days incubation. In contrast to these observations with aldolase, considerable subunit exchange between the H and M forms of LDH is evident after 30-min incubation in 0.02 M phosphate–0.1 M NaCl (pH 7–9) (Millar *et al.*, 1971), and exchange between rabbit and yeast TDH is observed after dialysis of the enzymes at 3 mg/ml for 14 hr at 4° (Spotorno and Hollaway, 1970).

A final experiment under nondenaturating conditions was performed in which uncentrifuged, crude extracts of chicken muscle (aldolase A) and brain (aldolases C and AC_3), prepared in 0.005 M dithiothreitol, were mixed and incubated at 40°. No subunit exchange between the aldolases was observed after 1 day.

EFFECT OF FREEZE-THAW ON ALDOLASE TETRAMERIC STRUCTURE. It is well known that hybridization between the parental H and M forms of LDH readily occurs after freeze-thaw of the enzymes in phosphate buffer and in high salt (Markert, 1963; Markert and Massaro, 1966; Chilson *et al.*, 1965). Although Tris appears to be an inhibitor of hybridization at high LDH concentrations, no such inhibition is observed at enzyme concentrations below 0.5 mg/ml (Markert and Massaro, 1968). Furthermore, high concentrations of halide ions are not required for hybridization when low concentrations (less than 0.5 mg/ml) of LDH are used (Markert and Massaro, 1968). To investigate the effect of freeze-thaw on aldolase, chicken aldolases A and C were mixed in equal proportions, diluted to 0.15–0.3 mg/ml, and frozen in polypropylene plastic tubes at −20°. After 48 hr, the solutions were thawed at room temperature. Greater than 80% recovery of activity, but *no subunit exchange* was observed by freezing and thawing the enzymes in 0.05 M Tris-HCl (pH 7.5) or 0.1 M sodium phosphate (pH 6.9) in the presence or absence of 1 M NaCl.

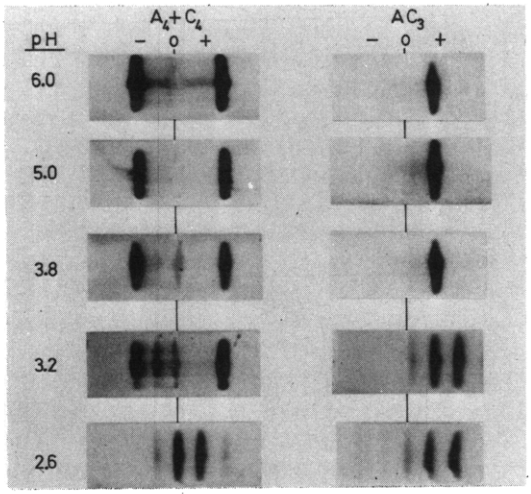

FIGURE 7: Effect of low pH on the quaternary structure of chicken aldolases; chicken aldolases A_4 plus C_4 and AC_3 at 0.1 mg/ml in 0.01 M Tris–HCl–0.001 M EDTA–0.01 M ME (pH 7.5) were placed in polypropylene plastic tubes (final volume 1 ml). The pH of the solutions were reduced to the indicated values by the addition of 0.25 N H_3PO_4. After incubation at 4° for 30 min, 0.5 ml of 1 M Tris-HCl (pH 8.0) was added and the neutralized solutions were subjected to electrophoresis.

EFFECT OF LOW pH ON ALDOLASE TETRAMERIC STRUCTURE. Using the criteria of fluorescence polarization, coenzyme binding, and ease of hybridization to study stability of quaternary structure, Anderson and Weber (1966) have demonstrated that hybrid LDH enzymes are more readily dissociated by acid than are the parental tetramers and have suggested that subunit–subunit interactions are strongest when all subunits of the LDH tetramer are of the same type. In view of these observations on LDH, it was of interest to compare the effect of decreasing pH on the tetrameric structure of parental and hybrid aldolases. Mixtures of chicken aldolases A and C or solutions of the asymmetrical hybrid AC_3 were brought to various hydrogen ion concentrations by addition of H_3PO_4. After 30 min at 4° the enzyme solutions were adjusted to pH 8 with 1 M Tris-HCl (pH 8.0) (see legend to Figure 7 for details). Little or no loss of activity was observed in those solutions which were maintained above pH 4 but only about 40% recovery of activity was obtained from those solutions treated at lower pH. As shown in Figure 7, no subunit exchange between the parental enzymes A_4 and C_4 was observed above pH 3.8 and A_3C was the only hybrid in significant amount detected after treatment at pH 3.2. Considerable dissociation of both parental enzymes occurred at pH 2.6 as evidenced by the predominance of hybrid molecules after neutralization. The greater intensity of aldolase C relative to A activity at pH 3.2 and the skewed profile of A-C hybrids toward the C terminus observed at pH 2.6 suggest that aldolase A or its subunits are less stable at low pH than are aldolase C or its subunits. The apparent instability of aldolase A in these experiments may be a reflection of the enzyme preparation itself since Deal and associates (1963) observed different stabilities to low pH by different aldolase A preparations. The intensity of A_3C in the pH 3.2 profile apparently reflects greater dissociation of A tetramers than C tetramers at this pH.

Similar experiments on the asymmetrical hybrid AC_3 did not reveal dissociation of this enzyme at pH 3.8 or above (Figure 7). Subunit exchange was detected at pH 3.2 and 2.6. Essentially results identical with those presented in Figure 7 were obtained when the enzymes were incubated at 22° for 30 min in 0.05 M citric acid–0.1 M Na_2HPO_4 buffers at the appropriate pH and then adjusted to pH 8 with 1 M Tris base. Although similar experiments on the other A-C hybrids have not yet been performed, the present results indicate that arrangement of aldolase subunits into hybrid tetramers does not render the quaternary structure of such tetramers less stable to low pH than that of the parental enzyme A_4.

Discussion

Aldolase Inactivation at Low-Enzyme Concentration. The present studies on isolated rabbit and chicken FDP aldolases demonstrate that these enzymes can be maintained at very low-protein concentrations for extended periods of time without loss of enzymatic activity. It follows, therefore, that reversible dissociation of active enzyme into inactive species, as previously proposed by Bernfeld *et al.* (1965), is not responsible for inactivation of these enzymes commonly observed at high dilution. Rather, it appears likely that interactions between aldolase molecules and vessel surfaces cause this inactivation. The protection against glass surface inactivation afforded by bovine serum albumin and other proteins is most likely due to saturation of glass "binding sites," thus precluding interactions between these sites and aldolase molecules. The observation that inactivation could not be reversed by subsequent addition of bovine serum albumin (or FDP) suggest that the aldolase molecules are inactivated at the vessel surface and not simply adsorbed to it. The protection afforded by preincubation of the glass tubes with dilute aldolase solutions suggests that, once inactivation occurs, the inactivated molecules remain for some time at the vessel surface.

In view of the apparent correlation between rate of enzyme inactivation and net positive charge on the various aldolase tetramers, it is possible that the mechanism of inactivation of these enzymes involves electrostatic attraction between positively charged aldolase subunits (A and B) and negatively charged glass surfaces; on the basis of electrophoretic and chromatographic behavior (Penhoet *et al.*, 1969a) and on direct isoelectric point measurements (see data by Susor *et al.*, 1969; Gurther and Leuthardt, 1970) mammalian aldolases A and B appear to be positively charged at neutral pH while aldolase C is negatively charged at this pH. If subunit charge is an important factor, then it would be predicted that other isoenzyme systems (for example, the M and H forms of LDH) may be expected to follow the same pattern of glass surface inactivation as found for the aldolases. Of course, other factors may be responsible for the different rates of inactivation seen with the various aldolases. For example, aldolase C subunits may themselves be more resistant to surface inactivation than are A and B subunits or may help to maintain the aldolase tetramer in a conformation which is more resistant to vessel surface interactions. However, the observation that the hybrid A_3C may be 100% inactivated by glass indicates that the catalytic activity of C subunits can be destroyed when present in hybrid combination. Alternatively, the observed resistance of the hybrid AC_3 suggests that A subunits may be protected when present in certain hybrid combinations. Lastly, inactivation of the aldolases may be influenced by factors arising during purification or treatment of the enzymes; variations in the rates of inactivation between freshly prepared and stored aldolase preparations were occasionally observed.

The observations that aldolase inactivation could be prevented or retarded, but not reversed, by low concentrations

of FDP suggest that binding of the negatively charged substrate to the enzyme's active center prevents those interactions between aldolase molecules and glass surfaces which normally lead to inactivation in the absence of substrate. This protection may involve stabilization of an active, resistant conformation of the enzyme; Lehrer and Barker (1971) have recently reported that a conformational change takes place upon binding of substrate to aldolase A. Clearly more detailed investigation is needed before the mechanisms of inactivation involved can be eluciated.

Structure of Catalytically Active FDP Aldolase. It is often difficult to appreciate what relationship, if any, exists between the quaternary structure of a multisubunit enzyme and expression of the enzyme's catalytic activity. This difficulty often arises due to the fact that the methods generally employed for molecular size measurements require protein concentrations several orders of magnitude higher than those used for measurements of catalytic activity. For example, in the case of aldolase, the sedimentation equilibrium ultracentrifugation data of Kawahara and Tanford (1966) suggest that rabbit aldolase A dissociates to dimers at enzyme concentrations below 0.2 mg/ml and, therefore, that the aldolase molecule exists largely as dissociated species under conditions of enzyme assay. It is possible, however, that these data reflect minor contamination of the commercial enzyme preparation used by these workers (specific activity = 11) since data from similar studies by Penhoet *et al.* (1969b) on several aldolases isolated from rabbit tissues, including the A enzyme (specific activity = 14–16), showed no decrease in molecular weight at concentrations as low as 0.1 mg/ml. The discrepancy between the data of Kawahara and Tanford (1966) and those of Penhoet *et al.* (1969b) as well as the observation that substrate can prevent dissociation of tetrameric aldolase A under some denaturating conditions (Masters and Winzor, 1971) emphasize the need to establish which molecular species of aldolase predominates under conditions of enzyme assay and what possible effect substrate may have on the quaternary structure of these enzymes.

The present studies have shown that the tetrameric species of parental and hybrid aldolases prevails at enzyme concentrations as low as 0.0002 mg/ml and that substrate is not required to maintain the tetrameric structure under these conditions. It follows, therefore, that the tetramer is the only active species of native mammalian and avian FDP aldolases under those conditions normally used to measure catalytic activity.

The tetrameric structure of catalytically active aldolase is also of interest from a functional point of view. The recent studies of Penhoet and Rutter (1971) as well as those of Meighen and Schachman (1970) suggest that the catalytic behavior of aldolase subunits is not affected by the catalytic properties of neighboring subunits in the tetramer. Indeed, the fact that a class I (Schiff base) (Rutter, 1964) FDP aldolase has been isolated (from the bacterium *Micrococcus aerogenes*) which has no quaternary structure[2] indicates that there is no absolute requirement for aldolase subunits to associate into oligomeric combinations in order to display catalytic activity. Although many of the catalytic properties of this and other class I aldolases are similar, structural studies, including partial characterization of the enzyme's active-site tryptic peptide, indicate that this bacterial aldolase may not be homologous to the class I aldolases of eucaryotes;[3] therefore, observations on this enzyme may not actually be applicable to the vertebrate aldolases. Finally, it has been suggested by Meighen and Schachman (1970) that stabilization of active conformations of the aldolase tetramer may depend on subunit–subunit interactions within the oligomeric molecule. Further studies on the extent of glass surface inactivation of A-C hybrid aldolases may help to clarify the role quaternary structure plays in the expression of catalytic activity of the vertebrate FDP aldolases.

Subunit Exchange as a Control Mechanism of Isoenzyme Levels within the Cell. If subunit exchange between multiple forms of enzymes occur *in vivo*, as has been suggested for LDH (Fritz *et al.*, 1971), such processes may also occur under mild *in vitro* conditions. Indeed, subunit exchange between LDH isoenzymes (Millar *et al.*, 1971; Fritz *et al.*, 1971) and between homologous forms of TDH (Spotorno and Hollaway, 1970) have been observed under *in vitro* conditions in which the enzymes remain totally active. In contrast, the present studies indicate that subunit exchange between the isolated mammalian and avian aldolases do not occur under similar mild conditions. The lack of detectable dissociation of parental and hybrid aldolases at low-protein concentration or at rather low pH (3.8) and the absence of hybrid formation after freezing and thawing parental aldolases in high salt, further indicate that the quaternary structure of these enzymes is of such stability as to preclude subunit exchange (dissociation) between aldolase tetramers under physiological conditions. Finally, the lack of exchange observed in crude tissue extracts suggests that particulate or other cell components are not required to facilitate subunit exchange between the aldolases. It must be emphasized however, that the negative results of the present studies do not eliminate the possibility that exchange between aldolase tetramers may occur under other mild *in vitro* conditions; or, more importantly, within the structurally intact cell.

Subunit exchange may play a role in the modulation of isoenzyme levels *in vivo*. Fritz and associates (1971) suggested that, in addition to phenomena associated with enzyme synthesis, degradative factors and subunit exchange between LDH molecules are involved in the control of LDH isoenzyme levels within the cell. In support of this concept, evidence is now available that different rates of degradation, as well as synthesis, of the H and M forms of LDH do occur in different mammalian tissues (Fritz *et al.*, 1969) and the observations that exchange between LDH tetramers occurs under mild *in vitro* conditions (see above) indicate that similar exchange processes may occur *in vivo*. By analogy, the ease of *in vitro* hybridization between homologous TDH molecules indicates that subunit exchange between the multiple forms of this enzyme found in some vertebrate tissues (Lebherz and Rutter, 1967) may also occur within the cell. It appears likely that, if subunit exchange occurs *in vivo*, the processes involved are highly dependent on the intracellular environment, including the levels of substrates and coenzymes. For example, there are reports that the association–dissociation equilibria of purified LDH (Hathaway and Criddle, 1966) and TDH (Hoagland and Teller, 1969) at low protein concentration is shifted toward the tetramer by the substrates of these dehydrogenases.

In vivo subunit exchange allows for an alternate route of hybrid molecule construction. It was previously assumed that

[2] H. G. Lebherz and W. J. Rutter, submitted to *J. Biol. Chem.*

[3] H. G. Lebherz, R. A. Bradshaw, and W. J. Rutter, submitted to *J. Biol. Chem.*

hybrid LDH enzymes were formed by the random association of H and M polypeptide chains from a subunit pool, the relative levels of each hybrid being dependent on the levels of each parental subunit in the pool (Markert and Ursprung, 1962). In contrast, Fritz *et al.* (1971) suggest that hybrid tetramers containing H and M subunits are not necessarily formed at the time of enzyme synthesis; rather, hybrids may be formed later as a result of subunit exchange between the H and M parental enzymes. If, as the present studies may indicate, *in vivo* subunit exchange between FDP aldolases does not occur, then the hybrid aldolases found in many vertebrate tissues must be formed at the time of subunit synthesis or subunit assembly. It appears likely that any pool of unassociated functional aldolase subunits within the cell must be exceedingly small since, at least *in vitro*, the tetrameric form of these enzymes is highly favored. Studies are currently in progress in the hope of elucidating the mechanisms involved in the control of aldolase isoenzyme levels *in vivo*.

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