Synthesis and Degradation of Fructose Diphosphate Aldolase Isoenzymes in Avian Brain*

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The intracellular proteins of animal cells are continuously turning over, therefore, the concentration of a given protein is regulated both at the level of protein synthesis and at the level of protein degradation. Studies on the relative rates of turnover of isoenzymes, such as those of aldolase and lactate dehydrogenase, may help to clarify the mechanisms involved in protein turnover. The isoenzymes and subunit types are very similar proteins, and are located within the same intracellular compartments; yet, the concentrations of these proteins are independently regulated.

The present paper describes the roles of synthesis and degradation in regulating aldolase isoenzyme concentrations in avian brain. (a) Steady state concentrations (approximately 700 μg/g) of aldolase are present in chicken brain virtually during the entire life of the organism, and the relative levels of the isoenzymes do not change with age. (b) The aldolase protein of this tissue, as determined by ion exchange chromatography, is made up of 5% A and 95% C subunits. The relative levels of tetramers present in whole brain extracts, C₄ (81%), A₄C₄ (17%), and A₄C₂ (2%) are quite similar to those predicted for the random association of 5% A and 95% C subunits. (c) Single and double isotope-labeling experiments using [3H]- and [14C]leucine as precursors of protein synthesis followed by isolation of the labeled isoenzymes by affinity chromatography or immunoprecipitation were performed to measure the relative rates of turnover of aldolases tetramers and subunit types. The results show that aldolases C₄ and AC₄ turn over at the same rates with apparent half-lives of about 4 days. Furthermore, a double isotope experiment showed that the A and C subunits of aldolase AC₄ turn over at the same rate. (d) Although these half-lives are overestimates due to reutilization of the labeled precursors during the experiments, two control experiments show that extensive isotope reutilization was not responsible for the calculation of essentially identical half-lives for the isoenzymes and subunit types. (The specific radioactivity of the free amino acid pool rapidly decreased after administration of [3H]leucine; and large differences in the rates of turnover of other brain proteins were demonstrated by the methods employed here.)

The present work suggests that the aldolases and subunit types have very similar rate constants for degradation and, therefore, that the degradative mechanisms of brain cells do not distinguish between isoenzymes or subunit types when selecting aldolase protein for degradation. It follows that, although regulation at the level of degradation is important in determining total amounts of aldolase protein, regulation at the level of subunit synthesis (transcription, translation) is responsible for determining the relative levels of A and C subunits, which generate the tissue-specific isoenzyme pattern of avian brain. These conclusions are discussed in relation to the model proposed by Fritz and associates on the regulation of lactate dehydrogenase isoenzyme concentrations in animal cells.

The intracellular proteins of animal cells are continuously being catabolized and resynthesized, a process known as turnover (3, 4). Although the detailed mechanisms involved in the turnover process are not well understood, several parameters have been shown to influence the rates of protein degradation. Among them are: the structural characteristics of the protein (3, 4), including subunit size (5, 6); the properties of the cells in which the protein is localized (3, 4, 7); and the composition of the intracellular milieu in which the protein is found (3, 8).

Investigations on the turnover of oligomers and subunits of multiple enzyme systems, such as the isoenzymes of lactate dehydrogenase, provide additional evidence for the involvement of degradation mechanisms in determining the concentrations of these proteins. The present work discusses the relationship between the rates of turnover and the relative levels of A and C subunits in avian brain, and suggests that regulation at the level of degradation is not the primary determinant of the tissue-specific isoenzyme pattern.
dehydrogenase and aldolase, may help to clarify the mechanisms involved in protein degradation. Using these systems, possible variations in the rates of turnover of different subunit types based on differences in gross conformation and primary structure, on differences in subunit size, or on differences in intracellular localization are eliminated, since the subunit types are structurally homologous, one of the same size, and are found in association with each other within the cell (2, 9-14).

Thus, any preferential selection of the isoenzymes or subunits for degradation would be determined by relatively minor structural and conformational differences, and/or by differences in abilities or inabilities to interact with the components of the cell.

Fritz and associates (15, 16) recently proposed that preferential degradation of different subunit types of lactate dehydrogenase is of major importance in regulating the relative levels of the isoenzymes in mammalian cells. Their model is of considerable interest, since it had previously been assumed (9, 17) that regulation at the level of transcription is primarily, if not solely, responsible for maintaining the well known tissue-specific isoenzyme patterns of animal cells. The model of Fritz et al. was a major stimulus to initiate the present studies on the in vivo turnover of aldolase isoenzymes and their subunits. Chicken brain was selected for study since it is a major vertebrate tissue which contains the easily separable isoenzymes belonging to the A-C hybrid set.

**EXPERIMENTAL PROCEDURE**

**Materials**

Newly hatched male white Leghorn chicks (obtained from Hallauer Zuchthall, Hallau, Switzerland) were maintained on Kurath chicken food. Other materials and their sources were: L-[4,5-3H]leucine (30 to 50 Ci/mmol) and L-[1-14C]leucine (342 mCi/mmol), Amersham; Aquasol for liquid scintillation counting, New England Nuclear; acrylamide, N,N' methylenebisacrylamide, and N,N,N',N'' tetrameth yl eth ylenediamine, Eastman; laboratory grade sodium dodecyl sulfate, Fisher; Sepaparohere III cellulose-polyacetate strips, Gelman; phosphocelluloses, Schleicher and Schuell; Linolens, DEAE A-50, G-25, and G-150, Pharmacia; substrates, coenzymes, and enzymes other than aldolases, Sigma and Boehringer. Aldolase isoenzymes were isolated from chicken tissue as previously described (18), or as described in the text. All other chemicals were of reagent grade.

**Methods**

**Analytical Methods**—Aldolase activity was determined at 25\(^\circ\) as previously described (19). Activities were expressed as micromoles of fructose-P\(_2\) cleaved per min per ml of enzyme solution, and specific activities as units of activity per mg of protein.

Protein concentrations of crude extracts, resolubilized trichloroacetic acid precipitates, and immunoprecipitates were determined by the method of Lowry et al. (20). Concentrations of pure aldolase solutions were measured by absorbance at 280 nm, using the extinction coefficients (0.9) of rabbit aldolase A\(_2\), and C\(_{12}\) (13, 21). Concentrations of aldolase solutions were estimated from activity measurements using specific catalytic activities of 16 and 8 for A and C subunit protein, respectively (18).

Cellulose-polyacetate electrophoresis and staining for aldolase activity were performed as previously described (22). Polycrylamide gel electrophoresis was done at pH 9.5 in 5% gels (23); electrophoresis was carried out with a current of 1.5 ma/gel and afterward, the gels were stained for proteins with 1% Amido schwarz or 0.25% Coomassie blue, followed by destaining in acetic acid/methanol/water, 1:1:8.

Tritium and 14C were counted in the Beckman LS-150 liquid scintillation system. Aquasol samples (0.9 to 9.0 ml) were counted in 10 to 13 ml of Aquasol. Trichloroacetic acid precipitates were dissolved in 1 N NaOH, and 0.4-ml aliquots were counted in 10 ml of Aquasol; prior to counting, the NaOH was neutralized by the addition of 0.6 ml of 1 N HCl. In single label experiments, 3H-label solutions were used to convert counts per minute to disintegrations per minute. Counting efficiencies were 25 to 35%. In double isotope experiments, [14C]leucine standards were counted to correct for 14C spillover (11 to 15%) into the 3H channel.

**Immunological Methods**—Antibodies toward aldolase C\(_2\) were produced in rabbits. By Dr. R. André. A solution (before containing approximately 150 \(\mu\)g of pure C\(_2\) was suspended in an equal volume of Freund's complete adjuvant, and 0.25-ml volumes were injected into each footpad of a rabbit. Three weeks later, 150 \(\mu\)g of enzyme were administered by multiple intramuscular injections. Two more injections of 75 \(\mu\)g each were administered at 1-week intervals. Two weeks after the last injection, blood was collected for serum production; the clear serum was stored at -20\(^\circ\).

Composite double diffusion tests were performed at room temperature in 0.8% Noble agar buffered with 10 mM Tris-Cl/4% NaCl, pH 7.5. Aldolase samples and antisera were adjusted to 4% NaCl. After incubation overnight, the gels were extensively washed with the above buffer to remove soluble proteins. The precipitin lines were then stained for protein with Amido schwarz or for aldolase activity with the staining solution (agar omitted) described by Penhoet et al. (22). See appropriate figure and table legends for additional immunological methods.

**Isolation of Brain Aldolases**—Whole brains from freshly killed chickens were homogenized in 5 volumes of Buffer I (10 mM Tris-Cl, 1 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.5) with a glass-Teflon homogenizer. After centrifugation at 17,000 \(\times\) g for 20 min, pellets were re-extracted in 5 volumes of Buffer I containing 0.2 M KC\(_1\) followed by centrifugation as before. KC\(_1\) was used in this second extraction to obtain a small, well defined pellet upon centrifugation.) Greater than 96% of the extractable aldolase activity was recovered using this method. Extracts were brought to 40% ammonium sulfate saturation (24.2 g/100 ml); after 1 hour, the precipitated proteins were collected by centrifugation at 16,000 \(\times\) g for 20 min and were discarded. Supernatants were brought to 60% saturation (15.1 g/100 ml) and, after 1 hour, precipitated proteins were collected as before. Precipitates were dissolved in Buffer I and the preparations equilibrated with this buffer by gel filtration on Sephadex G-50 columns. The aldolases were then isolated either by affinity chromatography or immunoprecipitation.

Affinity chromatography on phosphocellulose columns was performed by a modification of the method previously described (24). It was noticed that phosphoaldehydes obtained from Whatman and Schleicher-Schuell differed in their capacities to retain aldolase C\(_{12}\), and this distinction was used for the convenient separation of C\(_{12}\) and AC\(_{12}\) during the isolation procedure. Enzyme samples were applied to Whatman columns (1.8 cm \(\times\) 10 cm) equilibrated with Buffer I. The columns were washed until proteins not retained on the columns, including aldolase C\(_{12}\), were recovered. This material was applied to a Schleicher-Schuell column (1.8 cm \(\times\) 14 cm) equilibrated with Buffer I; here, the columns were washed with Buffer I, followed by Buffer I containing 1 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.0 until the A\(_{12}\) of the effluent was less than 0.02. The aldolases bound to the two columns (AC\(_{12}\), and C\(_{12}\), respectively) were then eluted with Buffer II containing 1 mM fructose-P\(_2\). Finally, columns were washed with buffer containing 2 M NaCl to elute other proteins.

Purification of aldolases AC\(_{12}\), and C\(_{12}\), by immunoprecipitation was performed after separation of the isoenzymes by ion exchange chromatography (see under "Results").

**Administration of Radioactive Leucine—After fasting overnight, chicks (110 to 140 g body weight) received [3H]- or [14C]leucine by intraperitoneal injection. Samples were administered in 50 mM sodium phosphate/150 mM sodium chloride, pH 7.2, between 8 and 9 a.m. in long term experiments, chicks were refed 3 hours after injection.

**RESULTS**

**Steady State Concentrations of Aldolase Isoenzymes in Chicken Brain**—A considerable fraction of the aldolase activity in certain tissues is not extractable with buffers of low ionic strength (2, 25). The particulate activity can be solubilized with high ionic strength buffers or with low ionic strength buffers containing certain metabolites (2, 25-28). In contrast to muscle or other tissues, which contain aldolases with large numbers of A subunits (2), all extractable aldolase of chicken brain (approximately 700 \(\mu\)g/g wet weight) could be solubilized with buffers of low ionic strength.
The relative amounts of the isoenzymes in brain extracts were determined by ion exchange chromatography on DEAE-Sephadex (Fig. 1). The levels of isoenzymes were found to be: C₄ (81%), AC₃ (17%), and A₅C₂ (2%). Only trace amounts of aldolases A₁C₁ or A₅ were ever detected. Since aldolase subunit associations are very stable in vitro (18), it may be assumed that the relative levels of isoenzymes observed were the same as those within the intact brain. From this tetramer distribution, it was calculated that A and C subunits comprise 5% and 95%, respectively, of the aldolase protein.

Brain tissue is quite heterogeneous (29) and, therefore, the isoenzyme content of whole brain extracts represents the sum contributions of all brain regions and cell types present in the intact tissue. Some differences in relative proportions of isoenzymes and in specific aldolase activity were observed when comparing different brain regions. For example, the ventral cerebrum contained relatively higher levels of A₂C₄ and lower levels of C₄ as compared with the other brain regions tested (medulla, optic lobe, cerebellum, and dorsal cerebrum).

Since aldolase subunits appear to associate in a random fashion (30), comparisons between the relative levels of isoenzymes observed in tissue extracts and those expected assuming random combination of subunits from a single subunit pool may give indications of the degree of cellular heterogeneity, with respect to isoenzyme content, of animal tissues (31). If observed and predicted values are the same, then tissue heterogeneity may not be a problem in interpreting turnover data. In the present studies, good agreement between observed and predicted values was obtained. However, since the isoenzyme distribution of chicken brain is highly skewed toward aldolase C₄ (Fig. 1), the possibility that significant differences in the isoenzyme patterns of the two predominant cell types of brain (neurons and glial cells) may exist could not be eliminated using such calculations. In view of these observations, cellular heterogeneity with respect to isoenzyme content must be considered in interpreting the turnover data described below, since the purpose here was to compare the rates of turnover of aldolase isoenzymes within the same cells.

There are advantages to performing the present turnover experiments on young chicks. Because of the smaller body weight and higher brain weight to body weight ratio, approximately 100-fold lower levels of radioactive amino acids are required to obtain the same extent of labeling of brain proteins of 2- to 3-week-old chicks as compared with those required for adult chickens. In using still developing animals, however, it must be demonstrated that steady state concentrations of the proteins under study are present at an early age. The relationship between aldolase isoenzyme content of brain and age (body weight) was therefore determined. As shown in Fig. 2, aldolase activity/g of brain has reached a constant maximal level prior to 1 week of age (body weight, 60 to 70 g). Specific catalytic activity has also reached the steady state level (0.145 to 0.155 unit/mg of soluble protein) by this time, and no changes in relative concentrations of the isoenzymes were indicated by electrophoresis. Thus, steady state concentrations of the isoenzymes have been accumulated in brain prior to 2 weeks of age, and young chicks were used for the turnover experiments described below. Since the brain did show considerable growth during the first 2 to 3 weeks after hatching, the brain weight versus body weight relationship in Fig. 2 was used to correct for increases in total aldolase content (brain mass) during the experiments.

Isolation of Brain Aldolase Isoenzymes—In the turnover experiments described below, aldolases AC₃ and C₄ were isolated by one of two purification procedures. In the affinity chromatography method, the aldolases were specifically eluted from phosphocellulose by fructose-P₃ (see “Methods” for details). Elution profiles of the Whatman (containing aldolase AC₃) and Schleicher-Schuell (containing aldolase C₄) phosphocellulose columns are shown in Fig. 3. The two aldolase preparations thus obtained were tested for purity by electrophoresis. Fig. 4 (upper) compares the activities in the two preparations with those in a crude brain extract. Polyacrylamide gel electrophoresis showed that the C₄ preparation was homogeneous, while the AC₃ preparation contained only small amounts of contaminating aldolases (A₂C₃ and C₄). Density gradient centrifugation of the gel showed that contamination of aldolase AC₃ by the other isoenzymes was usually about 10% (Fig. 4, lower).

In the immunoprecipitation method, aldolases AC₃ and C₄ were partially purified by ammonium sulfate fractionation (see “Methods”) and separated from each other by chromatography on DEAE-Sephadex, as described in Fig. 1. The isoenzymes were then specifically precipitated with antiserum directed toward aldolase C₄. Ouchterlony double diffusion tests with the antiserum are shown in Fig. 5. A single precipitin line was produced with crude brain extracts which completely fused with the lines produced with pure aldolases AC₃ and C₄. No precipitin line formed with crude muscle extracts (containing aldolase A₅). The antiserum, therefore, appears to be monospecific for aldolase C subunit protein, and recognizes.

![Fig. 1. Separation of chicken brain aldolases on DEAE-Sephadex.](image)

Two grams of fresh chicken brain were homogenized in 4 volumes of 10 mM Tris Cl, 1 mM EDTA, and 1 mM 2 mercaptoethanol, pH 7.5. After centrifugation at 17,000 × g for 30 min, the supernatant fraction was applied to a DEAE-Sephadex A-50 column (1.4 cm × 35 cm) equilibrated with the above buffer but containing 0.1 M NaCl and 0.1 mM fructose-P₃. The column was developed with a linear 0.1 M to 0.45 M NaCl gradient (400 ml total). Greater than 90% recovery of activity was obtained using specific catalytic activities of 16 and 8 units/mg, respectively, for A and C subunits.

![Fig. 2. Relationship between brain aldolase content and age.](image)

The body weights of chicks of various ages were determined and the brain aldolases were extracted as described under “Methods.”
Fig. 3. Isolation of aldolases AC₃ and C₄ by substrate elution from phosphocellulose. The brain aldolases were partially purified as described under "Methods." The aldolase preparation was applied to a Whatman (upper) phosphocellulose column (1.8 cm x 10 cm) and the large breakthrough peak (containing aldolase C₄) was applied to a Schleicher-Schuell (lower) column (1.8 cm x 14 cm). The two columns were developed as described under "Methods." The aldolases were eluted with fructose-P₅ (B) and remaining proteins were eluted with 2 M NaCl (C) (see "Methods" for details).

Fig. 4. Purity of brain aldolase preparations. Aldolases AC₃ and C₄ were isolated as described in Fig. 3. Upper, cellulose polyacetate electrophoresis followed by staining for aldolase activity. a, Crude brain extract; b, aldolase AC₃; c, aldolase C₄. Lower, polyacrylamide gel electrophoresis. The gels were stained for protein with Amido schwarz and were then scanned at 610 nm. - - - , AC₃ preparation; -- -- - - , C₄ preparation.

Fig. 5. Characterization of rabbit antiserum directed toward chicken aldolase C₄. Upper, Ouchterlony double diffusion tests. Outer wells contain crude brain (B), crude muscle (M), or purified aldolases, as indicated. Aldolase concentrations in all preparations were between 0.15 and 0.20 mg/ml. The precipitin lines were stained for protein or for activity as described under "Methods." Lower, immunotitration of chicken brain extracts. Tubes contained 0.5 ml of antiserum and increasing volumes of diluted brain extracts. All volumes were adjusted to 3 ml with 10 mM Tris-Cl, and all samples contained 4% NaCl. The tubes were incubated at room temperature for 1 hour and overnight at 4°. After centrifugation at 3000 x g for 15 min, the supernatant fractions were assayed for aldolase activity. The precipitate fractions were washed in the above buffer, including 4% NaCl and assayed for protein as described under "Methods."

AC₃ and C₄ with apparent immunological identity. The antigen-antibody complexes are enzymatically active, as shown by direct staining of the precipitin lines for aldolase activity (Fig. 5, upper right). A typical immunotitration curve using the antiserum and crude brain extract (Fig. 5) showed that each ml of antiserum precipitated approximately 0.54 unit (67 μg) of brain aldolase. Control experiments, in which unlabeled aldolase C₄ was added to [³H]leucine-labeled liver extracts (containing labeled aldolase B₄), followed by immunoprecipitation with the antiserum and washing of the immunoprecipitate, showed that all radioactivity was removed from the antigen-antibody complexes. Therefore, the antiserum could be used to precipitate specifically aldolases containing C₄ subunits from solutions containing mixtures of proteins.

Time Course of [³H]Leucine Incorporation into Aldolases AC₃ and C₄. The rates of turnover of aldolases AC₃ and C₄ in chicken brain were first investigated by measuring the time courses and extents of labeling of the isoenzymes after administration of [³H]leucine. As shown in Fig. 6, incorporation of isotope into both aldolases followed similar time courses with incorporation into both enzymes tapering off after about 2 hours. Taken alone, this observation suggests that the two isoenzymes turn over at similar rates, since the time course of isotope incorporation is related to the rate of protein turnover.
However, the fact that AC₄ became labeled to a considerably higher extent than C₄ in itself, suggests that AC₄ turns over more rapidly than C₄. Since A and C subunits contain approximately the same number of leucyl residues per polypeptide chain (33, 34), the greater extent of labeling of aldolase AC₄ cannot be explained as reflecting a higher specific activity of leucine in A as compared to C subunits. One possible explanation for the conflicting data of Fig. 6 may be that the isoenzymes turn over at similar rates, but that different portions of the different isoenzymes were derived from different cell types (see above). In this case, differences in the specific radioactivity of leucine at the sites of protein synthesis in the different cell types may be responsible for the different extent of labeling of the two aldolases.

Turnover of Aldolases AC₃ and C₄—The rates of turnover of aldolases AC₃ and C₄ were next investigated by measuring the decline in specific radioactivity of the isoenzymes after administration of [3H]leucine. Since difficulties in the isolation of aldolase C₄ at some times were encountered, reliable data for the turnover of this isoenzyme were not obtained in this experiment. The data for AC₃ are given in Fig. 7. As expected from what is known about protein degradation in animal cells (3-5), the decline in specific radioactivity followed first order kinetics. The open circles show the data prior to correction for increase in brain mass during the experiment, while the closed circles show the same data after correction for the increase in brain mass (i.e. total aldolase activity), using the brain weight versus body weight relationship of Fig. 2. From the slope of the corrected curve, an apparent half-life of 4.2 days was calculated for aldolase AC₃.

The relative rates of turnover of aldolases AC₂ and C₄ were then determined using the double isotope method described by Arias et al. (35). Using this method, relative rates of turnover of proteins can be accurately determined in a single animal. The animal receives one isotopic form of an amino acid (in this case, [3H]leucine) followed some time later by the same amino acid labeled with another isotope ([14C]leucine). The proteins are then isolated and their 14C:3H ratios determined. Since proteins which turn over rapidly are synthesized faster and degraded faster than proteins which turn over more slowly, the former will have higher 14C:3H ratios than the latter (35). Also, any differences between the ratios of the proteins will be increased as the time interval between administration of the two isotopes is increased; no differences in the ratios would be observed if the isotopes were administered simultaneously.

In the first series of experiments, chicks received [3H]leucine followed either 2, 5, or 9 days later by [14C]leucine. One day after the last injection, the brain aldolases were isolated by affinity chromatography, and their 14C:3H ratios determined. The data are given in Table I. Since different amounts of isotope were given to each group of chicks, the important values here are the normalized ratios for the two isoenzymes isolated from each group. These values are quite close to those expected for proteins that turn over at the same rate (1.00). For comparison, note the very different values that are predicted assuming that aldolase AC₃, with an apparent half-life of 4.2 days, turns over twice as fast as aldolase C₄ (last column).

Purification of the isoenzymes by affinity chromatography results only in the isolation of functional aldolase tetramers (tetramers capable of binding substrate) and, therefore, the experiments described above were concerned with the turnover of active aldolase molecules. Another double isotope experiment was performed to determine the relative rates of turnover of the isoenzymes using immunological competence as the criterion for aldolase isolation.

The data at the bottom of Table I suggest that immunologically competent forms of aldolases AC₃ and C₄ also turn over at similar rates.

Turnover of A and C Subunits of Aldolase AC₃—It is now known that subunits which comprise the same oligomeric structure may turn over independently of each other (36-38). Therefore, if aldolase A and C subunits turn over independently and at different rates, the measured rate of turnover of aldolase AC₃ would be largely a measure of the turnover of C subunits. This is apparent from the subunit composition of aldolase AC₃. Consider the situation (Fig. 8) in which it is assumed that C subunits, whether present in homo- or heterotetramers, turn over half as fast as A subunits. The apparent half-life of AC₃ (4.2 days) would be quite close to that of C₄ (4.8 days), while the differences in half-lives of A (2.4 days) and C (4.8 days) subunits would be readily discernible (Fig. 8). A double isotope experiment was performed to clarify this point.

![Fig. 6. Time course of [3H]leucine incorporation into aldolases AC₃ and C₄ of chicken brain. Chicks received 174 µCi of [3H]leucine/100 g of body weight. At the times indicated, two chicks were taken, and the brain aldolases were isolated by affinity chromatography.](image)

![Fig. 7. Rate of turnover of chicken brain aldolase AC₃. Chicks received 80 µCi of [3H]leucine/100 g body weight and at the times indicated, the brains from two chicks were taken for analysis. Aldolase AC₃ was isolated by affinity chromatography, and the disintegrations per min/mg of protein determined from catalytic activity and radioactivity measurements (see "Methods"). \( O - O \), data uncorrected for increase in brain mass during the experiment; \( O - O \), data corrected for increase in brain mass using the brain weight versus body weight relationship of Fig. 2.)

Downloaded from http://www.jbc.org/ on March 21, 2020
obtained from the Whatman Co.

...were then counted.

aldolase C, appear to reflect batch differences in the phosphocellulose...tetramers (A,A* and A&C*, respectively) during the reassociation process. The two tetramers containing the labeled subunits of aldolase AC, were essentially identical, demonstrating that the two subunit types turn over at similar rates.

...were detected after reassociation demonstrates the complete randomization of subunit associations as a result of the pH 2.3 treatment. No cross-contamination of the A, and A,C preparations, and hence, of labeled A and C subunits, was indicated by electrophoresis (Fig. 10). The two subunit preparations were treated as described in Table II and were then counted.

As shown in Table II, the 14C:3H ratios of the A and C subunits of aldolase AC, were essentially identical, demonstrating that the two subunit types turn over at similar rates.

Isotope Reutilization—Determinations of the rates of protein turnover using the long term single and double isotope methods employed here were overestimated due to isotope reutilization, since some of the labeled amino acids liberated during protein turnover were reutilized by the animal. As shown in Fig. 9, considerable contamination of the AC, preparation by aldolase C, occurred. The 14C:3H ratios of the two isoenzyme preparations (0.89) were found to be identical. The A and C subunits of the AC, preparation were then separated as follows. The labeled aldolase was added to a 39-fold molar excess of unlabeled aldolase A, and the tetratomers were dissociated to subunits by titration to pH 2.3 (18, 30, 39, 40). Reassociation to tetramers was then effected by titration to pH 8 with complete recovery of aldolase activity (see legend of Fig. 10 for details). The gels were stained with Coomassie blue and scanned at 570 nm to determine the relative levels of the isoenzymes in the aldolase AC, preparation.

FIG. 9 (left). Polyacrylamide gel electrophoresis of purified brain aldolase preparations. Five chicks received [3H]leucine followed by [14C]leucine as described in the legend of Table II. The brain aldolases were isolated by affinity chromatography and subjected to electrophoresis as described under "Methods." The gels were stained with Coomassie blue and scanned at 570 nm to determine the relative levels of the isoenzymes in the aldolase AC, preparation.

FIG. 10 (right). Separation of A and C subunits of aldolase AC, Dissociation-Reassociation—The labeled aldolase AC, preparation (see Fig. 9) containing 0.65 mg of protein was added to a solution containing 25 mg of unlabeled aldolase A,. The sample was diluted to 40 ml with water, and was adjusted to 0.1 M 2-mercaptoethanol. The tetratomers were dissociated to subunits at pH 2.3 by the dropwise addition of 1 M citric acid to pH 2.3. After 30 min, reassociation to tetratomers was affected by the dropwise addition of the sample to 360 ml of 0.01 M Tris-Cl, 1 mM EDTA, 0.05 M 2-mercaptoethanol, pH 8. Molar Tris base was added, when necessary, to maintain pH 7.7 to 8.0. The solution was then stirred at room temperature for 1 hour. Complete recovery of aldolase activity was obtained after reassociation. The sample was concentrated to approximately 1.5 mg/ml of protein by ultrafiltration with Diaflo PM 30 membranes.

DEAE-Sephadex A-50 Chromatography—The reassociated enzyme solution was applied to a DEAE-Sephadex A-50 column (1.4 cm x 6 cm) equilibrated with 0.01 M Tris-Cl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 mM fructose-P, pH 7.8. The column was washed with this buffer until all of the aldolase A, (containing labeled A subunits) was eluted. Aldolase A,C (containing labeled C subunits) was then eluted with buffer containing 0.25 M NaCl. Fractions were pooled as indicated, and the preparations were tested for purity by cellulose-polyacetate electrophoresis.

As shown in Table II, the 14C:3H ratios of the A and C subunits of aldolase AC, were essentially identical, demonstrating that the two subunit types turn over at similar rates. Specific radioactivities of both isoenzymes were normalized to those expected 1 day after isotope administration.
counted in 10 ml of Aquasol. Externally dialyzed against 1 mM 2-mercaptoethanol, and then lyophilized to dryness. Residues were taken up in 1 ml of water and adjusted to equal protein concentration (0.7 mg/ml) by addition of bovine serum albumin. The preparations were extensively dialyzed against 1 mM 2-mercaptoethanol, and then lyophilized to dryness. Residues were taken up in 1 ml of water and counted in 10 ml of Aquasol.

Relative rates of turnover of A and C subunits of chick brain aldolases

Five chicks each received 500 μCi of [3H]leucine followed 6 days later by 82 μCi of [14C]leucine. On the 7th day, the brain aldolases were isolated by affinity chromatography. A and C subunits of the aldolase AC, preparation were separated as described in Fig. 10. The two subunit preparations were diluted to equal volumes (19 ml) with 1 mM 2-mercaptoethanol, and adjusted to equal protein concentration (0.7 mg/ml) by addition of bovine serum albumin. The preparations were extensively dialyzed against 1 mM 2-mercaptoethanol, and then lyophilized to dryness. Residues were taken up in 1 ml of water and counted in 10 ml of Aquasol.

| Subunit | TCH | TCC | TCH/TCC | Normalized 14C:3H |
|---------|-----|-----|----------|-------------------|
| A       | 523 | 466 | 0.891    | 1.05 1.00         |
| C       | 1000| 850 | 0.850    | 1.00 1.00         |

* Normalized ratios predicted, assumed that A and C subunits turn over at identical rates.

degradation will be utilized as precursors of protein synthesis (41). For example, compare the apparent half-life of hepatic catalase (about 4 days), as determined by the rate of decline in specific radioactivity after administration of [3H]leucine, with the true half-life of the protein (1.8 days) as determined by the reappearance of catalytic activity after irreversible enzyme inhibition (42-44). If extensive reutilization of tritium occurred during the present experiments, differences in the relative rates of turnover of the aldolase isoenzymes might not have been detected, even if differences existed.

To investigate the turnover of brain proteins, several experiments were performed. In most of these studies, the problems of isotope reutilization were not considered. Furthermore, the report by Stewart and Urban (45) that the cellular amino acid pool of mouse brain remained highly labeled for a long time after administration of [3H]leucine suggests that isotope reutilization in brain cells might be quite extensive. Consequently, control experiments were needed to show that the similar 14C:3H ratios of the aldolases and subunit types do reflect similar rates of turnover of these proteins.

First, the approximate time courses of disappearance of tritium from the total, trichloroacetic acid-soluble, and trichloroacetic acid-insoluble fractions of brain extracts (17,000 × g supernatant fraction) were measured. As shown in Fig. 11, the tritium in the trichloroacetic acid-soluble and -insoluble fractions were nearly equal at all times after [3H]leucine administration. However, when the soluble fractions were lyophilized, the tritium counts in the residues rapidly decreased with time. Less than 3% and 0.3% of the trichloroacetic acid-soluble counts were found in the residues after 1 and 7 days, respectively (Fig. 11); the volatile material is presumed to be [3H]water, since Banker and Cotman (46) have recently shown that tritium of [3H]leucine is rapidly metabolized to [3H]water by mouse brain. Thus, the radioactivity in the cellular amino acid pool of tritium in the cellular amino acid pool decreased fairly rapidly after administration of [3H]leucine.

Second, the relative rates of turnover of soluble brain proteins were investigated. This control experiment is more meaningful than the one presented above, since it appears that amino acids are compartmentalized within the cell (47-49). Consequently, measurements of the radioactivity in the total cellular amino acid pool may not reflect the specific radioactivity and turnover of amino acids that serve as precursors of protein synthesis at the site(s) of protein synthesis; these precursors may actually bypass the large cellular amino acid pool altogether (48). Isotope reutilization should influence apparent half-lives of all soluble brain proteins, and, therefore, the detection of large differences in the relative rates of turnover of the soluble brain proteins would strengthen the argument that the brain aldolases do, in fact, turn over at similar rates. This was accomplished by an experiment which is analogous to those used to demonstrate a correlation between protein subunit size and rate of protein turnover in other systems (5, 6, 50, 51).

One chick received [3H]- and [14C]leucine simultaneously, and another received the isotopes 6 days apart. On the 7th day, the soluble brain proteins were dissociated to subunits with sodium dodecyl sulfate, and the subunits were separated according to size by gel filtration. The results are shown in Fig. 12. As expected, no consistent differences in the 14C:3H ratios of the protein subunits were observed when the isotopes were administered simultaneously. However, large differences in the 14C:3H ratios were apparent when the isotopes were given 6 days apart and, as expected, larger subunits had higher ratios than smaller ones. Thus, differences in the rates of turnover of brain proteins were readily detected with the procedures employed here.

**Discussion**

The present studies show that steady state concentrations of aldolase isoenzymes are present in chicken brain virtually throughout the entire life of the organism, and that the aldolase

**FIG. 11.** Kinetics of disappearance of tritium from the total, trichloroacetic acid-soluble, and trichloroacetic acid-insoluble fractions of brain extracts (17,000 × g supernatant). Chicks received 35 μCi of [3H]leucine/100 g body weight. At the times indicated, the brain from one chick was homogenized in 10 volumes of water, and the homogenate centrifuged at 17,000 × g for 30 min. For protein precipitation, 1/3 volume of 50% trichloroacetic acid was added to brain extract and, after 4 hours at 0°C, the soluble and insoluble material was separated by centrifugation at 3000 × g for 10 min. The soluble fraction was extracted three times with 2 volumes of ether to remove the trichloroacetic acid. Aliquots of the aqueous phase were lyophilized to dryness and the residues taken up in water. The precipitates were washed by suspension in 6 to 8 volumes of 10% trichloroacetic acid containing 5 mM L-leucine, and the precipitates were recovered by centrifugation as before. The washing procedures were repeated two more times, and the final pellet was dissolved in N NaOH. All brain fractions were counted for tritium content as described under "Methods."
Turnover experiments were performed to determine if regulation at the level of protein degradation was involved in maintaining the highly different steady state concentrations of the subunit types. Since brain is quite heterogeneous, both with respect to its regional and cellular composition, the measured relative rates of turnover of aldolase tetramers (AC₂ and C₄) do not necessarily reflect the turnover of different isoenzymes within the same cell. However, the observation that A and C subunits of the AC₂ heterotetramer turn over at the same rate does reflect the turnover of the subunits within the same cell(s); the subunits must have been associated with each other in the intact tissue, since rearrangement of aldolase subunit associations does not occur in tissue homogenates or during aldolase purification (18). Thus, these data suggest that the degradative mechanisms of brain cells do not distinguish between A and C subunits when selecting aldolase protein for degradation.

The present work defines the roles of synthesis and degradation in regulating aldolase isoenzyme concentrations in avian brain. Although the isoenzymes and subunit types are continuously being degraded and resynthesized, no net change in amounts of these proteins occurs with time. This observation necessitates that the rate of synthesis (Vₛ) of each protein is equal to its rate of degradation (V₇). Synthesis follows pseudo-zero order kinetics, so Vₛ can be expressed in terms of a zero order rate constant for synthesis (Kₛ). Degradation follows pseudo-first order kinetics, and V₇ can be expressed as the product of a first order rate constant for degradation (K₇) and the steady state level (P) of the isoenzyme or subunit type (3, 4). Thus, the steady state level (P) = Kₛ/K₇, where K₇ = ln 2/t₁/₂ (3, 4). Since the half-lives of the isoenzymes and subunit types calculated here (4.2 days) are surely overestimated due to isotope reutilization (see “Results”), no attempt was made to define numerical values for Kₛ and K₇. The important question concerns the relative rates of turnover of the subunits and isoenzymes. Since aldolase A and C subunits apparently have the same rate constants for degradation, and since the concentration of C subunits is 19-fold higher than that of A subunit, it follows that, per unit of time, 19 C subunits are degraded and resynthesized for every A subunit which turns over. It can be calculated from the present work (Table I and Fig. 8) that at least 1 x 10⁴ A and 19 x 10⁴ C subunits are synthesized and destroyed per gram of brain each day. Although regulation at the level of degradation is involved in determining total aldolase protein concentration, it is suggested that regulation at the level of subunit synthesis (transcription, translation) is predominantly, if not solely, responsible for determining the relative levels of aldolase isoenzymes and subunit types in the avian brain. Whether or not this conclusion can be generalized to explain the regulation of aldolase isoenzyme concentrations in other tissues, or in tissues of other organisms must await the outcome of additional experiments.

In contrast to the above emphasis on regulation at the level of subunit synthesis for the control of aldolase isoenzyme concentrations, Fritz and associates have proposed that regulation at the level of degradation is of major importance in controlling the relative concentrations of lactate dehydrogenase isoenzymes in mammalian cells (15, 16). Their reports that lactate dehydrogenase B subunits turn over very much faster than A subunits in skeletal muscle, while A subunits turn over faster than B subunits in cardiac muscle, pointed to a high degree of specificity in the selection of subunit types for degradation, and suggested that this specificity was determined by the characteristics of the cells in which the isoenzymes were localized. However, an entirely different interpretation of their data has recently been published (31), which suggests that the nonrandom distribution of lactate dehydrogenase subunits and differential turnover of the isoenzymes reported by these workers (15, 16, 52, 53) may simply reflect cellular heterogeneity, with respect to isoenzyme content, of the tissues studied; different rates of turnover of lactate dehydrogenase in different cell types of the same tissue would not be unexpected, since rates of turnover of proteins are partially determined by the properties of the cells in which the proteins are localized (3, 4, 7). Consequently, it is not yet known whether or not mechanisms other than those that control rates of subunit synthesis are involved in the regulation of relative concentrations of the isoenzymes within the same cell.

Correlations between rates of protein turnover in vivo and susceptibility to proteolytic inactivation and digestion in vitro has been observed (6, 54, 55). Also, it has been suggested that rates of protein turnover may be determined by the rates at which proteins spontaneously denature within the cell (56). If these observations and suggestions truly bear on the turnover process, then it would not be totally unexpected to find different isoenzymes of aldolase or lactate dehydrogenase turning over at different rates within the same cell. For example, different isoenzymes of lactate dehydrogenase show different susceptibilities to proteolytic inactivation in vitro, and with some isoenzymes, this susceptibility may be modified by the coenzyme NAD (57). Also, different isoenzymes of aldolase and lactate dehydrogenase (58) show different sus-
ceptibilities to heat denaturation and, in the case of lactate dehydrogenase, certain metabolites protect some isoenzymes from denaturation, but not others (58). Meaningful correlations between rates of degradation of isoenzymes in vivo and the behavior of isoenzymes in vitro may yet be found. If so, such correlations may help to clarify the mechanisms involved in intracellular protein degradation.

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