The Comparative Enzymology of Lactic Dehydrogenases. II. Properties of the Crystalline HM_3 Hybrid from Chicken Muscle and of H_2M_2 Hybrid and H_4 Enzyme from Chicken Liver*

THOMAS P. FONDY,† AMADEO PESCE,‡ IRWIN FREEDBERG,§
FRANCIS STOLZENBACH, AND NATHAN O. KAPLAN

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass.[||]

Received November 7, 1963

The hybrid lactic dehydrogenases H_2M_2 from chicken liver and HM_3 from chicken leg muscle have been isolated and crystallized. The pure H_4 form of the enzyme has been isolated and crystallized from chicken liver and compared to the H_4 obtained from heart. Fingerprint patterns of tryptic digests of the hybrids have been compared to patterns obtained from a 1:1 mixture of the H_4 and M_4 and have shown the hybrids to be a combination of the pure forms. Amino acid analyses, particularly of histidine, place the hybrids at points intermediate between the pure types. The H_4 from chicken liver did not vary significantly in amino acid content or molecular weight from the H_4 obtained from chicken heart. Molecular weights of the hybrids were in the same range as those of the pure types. Heat and time stability studies of the chicken hybrids and of beef hybrids isolated from starch grain have established the intermediate nature of the hybrids. Immunological properties, analog ratios, and oxalate inhibition also demonstrate that the hybrids are combinations of the H_4 and M_4 forms of the enzyme.

The evidence set forth on the developmental characteristics of lactic dehydrogenase in chicken (Cahn *et al.*, 1962) and in mammals (Fine *et al.*, 1963) has indicated that the lactic dehydrogenases of these animals are composed of two types of subunits which may be combined to yield five electrophoretically distinguishable enzymes. The lactic dehydrogenase of beef heart has been dissociated in the presence of 5 M guanidine into subunits of approximately 35,000 mw (Appella and Markert, 1961). The beef heart and chicken heart enzymes similarly have been dissociated with sodium dodecylsulfate (Di Sabato and Kaplan, 1964). The intermediate lactic dehydrogenases have recently been made *in vitro* by incubation of the extreme electrophoretic types (Markert, 1963).

In this paper we shall refer to the subunits of lactic dehydrogenase either as H or M type and to the native enzymes as H_4, H_3M, H_2M_2, HM_3, and M_4 in order of their decreasing mobility toward the anode at *p*H 7.0. This paper is concerned with the isolation and characterization of two of the hybrid lactic dehydrogenases, HM_3 and H_2M_2, from chicken tissues. These characterizations are compared with the results for the M_4 and H_4 types. Analysis of the amino acid compositions and "fingerprint" patterns of these enzymes yielded results consistent with the hypothesis that these enzymes consist of two distinct polypeptide subunits.

H_4 from chicken liver was isolated and characterized to determine whether this enzyme was distinguishable from that isolated from chicken heart. The chicken was chosen for this study because preliminary investigations of the H_4 and M_4 enzymes had shown significant differences in amino acid compositions and permitted the easy identification of these enzymes upon total amino acid analysis (Pesce *et al.*, 1964).

Materials and Methods

Starch-Gel Electrophoresis.—Gel electrophoresis followed the procedures set forth elsewhere (Kaplan and Cahn, 1962; Fine *et al.*, 1963; Fine and Costello, 1963).

Preparation of H_2M_2 and H_4 from Chicken Liver.—Step 1. Crude Extract.—Twenty lb of fresh chicken livers was ground three times in a mechanical meat grinder. The minced liver was then suspended in 6 liters of cold distilled water for 45 minutes and stirred occasionally. The mince was filtered through cheesecloth and resuspended in 4 liters of cold distilled water for an additional 45 minutes and occasionally stirred. After the washed mince was again passed through cheesecloth it was discarded, and the two filtrates were combined. The combined filtrates were clarified in a refrigerated centrifuge at 1300 × *g* for 30 minutes and the residue was discarded.

Step 2. First Ammonium Sulfate Precipitation.—Solid ammonium sulfate was added to 70% saturation[1] and the suspension was left at 4° for several hours and then filtered overnight on fluted filter papers. The precipitate was scraped off and dissolved in 1 liter of cold distilled water and dialyzed against two changes of cold distilled water (20 liters), about 6 hours for each change.

Step 3. Second Ammonium Sulfate Precipitation.—After dialysis, the enzyme suspension was centrifuged at 20,000 × *g* for 10 minutes. To the clear supernatant, solid ammonium sulfate was added to give 40% saturation. This suspension was left at 0° for 1 hour and centrifuged at 18,000 × *g* for 30 minutes. The major activity remained in the supernatant. Ammonium sulfate was then added to the supernatant to give a 60% saturation. After 1 hour at 0°, the

* Supported in part by grants from the American Cancer Society (P77-F) and the National Institutes of Health (CA-03611).

† Postdoctoral fellow under National Institutes of Health Training Grant 5TINB-05241-05.

‡ Predoctoral Public Health Service Fellow (grant 17680).

§ Postdoctoral fellow under National Institutes of Health Training Grant 5Tl-GM-0212-05. Present address: Dept. of Dermatology, Harvard Medical School at the Beth Israel Hospital, Boston, Mass.

|| Publication No. 280 of the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass.

[1] Per cent saturation was based on Table I in S. P. Colowick and N. O. Kaplan (eds.), *Methods in Enzymology, Vol. I.*, New York, Academic Press (1955), p. 76, even though the enzymatic solutions were kept at 4°.

precipitate was centrifuged as before and dissolved in cold distilled water. After centrifugation, the dissolved precipitate was dialyzed overnight against 20 liters of cold distilled water.

Step 4. Acetone Fractionation.—The dialyzed preparation from step 3 was brought to a concentration of 20% acetone by the addition of 100% acetone which had been precooled to −15°. This and the following acetone fractionation were carried out at −5°. After standing for 10 minutes at −5°, the precipitate was collected by centrifugation and discarded. Enough acetone was added to the supernatant to raise the concentration to 40% acetone. After 10 minutes at −5°, the precipitate was collected by centrifugation and suspended in 5×10^{-3} M Tris, *p*H 7.6. The insoluble material was centrifuged off and discarded.

Step 5. Refractionation with Ammonium Sulfate.—Enough ammonium sulfate was added to raise the concentration to 30% saturation. After standing at 0° for 30 minutes, the precipitate was centrifuged out and discarded. Ammonium sulfate was added to the supernatant to 60% saturation. The precipitate that was obtained was centrifuged, dissolved in 5×10^{-3} M Tris, *p*H 7.6, and dialyzed overnight against the same buffer.

Step 6. Crystallization and Purification of H_4.—Ammonium sulfate was added to 31% saturation and the precipitate was quickly collected by centrifugation and discarded. To the supernatant ammonium sulfate was added slowly to 40% saturation. Protein crystals appeared and were harvested and identified as H_4 and H_3M lactic dehydrogenase. These crystals were recrystallized by solution in water and addition of ammonium sulfate to 39% saturation. The recrystallized material was purified on a DEAE-cellulose column prepared as outlined elsewhere (Pesce *et al.*, 1964). A gradient of 1 liter of 1×10^{-1} M Tris, *p*H 7.6, flowing from above into 1 liter of 5×10^{-3} M Tris, *p*H 7.6, was used to elute the enzyme. The enzyme fraction was recrystallized in 35% ammonium sulfate. This lactic dehydrogenase fraction showed a mobility on starch-gel electrophoresis identical to the mobility of the H_4 enzyme isolated from chicken heart.

Step 7. Crystallization of H_2M_2 and H_3M Hybrid.—The supernatant from the 40% ammonium sulfate crystallization of H_4 and H_3M was brought to 60% saturation by the addition of ammonium sulfate. The precipitate consisted of mixed H_3M and H_2M_2 types of lactic dehydrogenase. This precipitate was dissolved and dialyzed against 5×10^{-3} M Tris, *p*H 7.6.

Step 8. DEAE-Cellulose Chromatography.—The dialyzed solution was then placed on a DEAE-cellulose column (4 × 30 cm) prepared as described in paper I of this series (Pesce *et al.*, 1964). A gradient of 1 liter of 10^{-1} M Tris, *p*H 7.6, flowing from above into 1 liter of 5×10^{-3} M Tris, *p*H 7.6, was used. The first peak of lactic dehydrogenase activity proved to be mainly the H_2M_2 form. The tubes showing this activity were combined and dialyzed against saturated ammonium sulfate to concentrate the enzyme. The precipitated enzyme was centrifuged and dissolved in 5×10^{-3} M Tris, *p*H 7.0, and dialyzed against the same solution.

Step 9. Carboxymethyl-Cellulose Chromatography.—The dialyzed enzyme was placed on a carboxymethyl-cellulose column (4 × 30 cm) prepared as described elsewhere (Pesce *et al.*, 1964). The pure H_2M_2 form of lactic dehydrogenase was eluted off the column with a gradient of 1 liter of 10^{-1} M Tris, *p*H 7.0, flowing from above into 1 liter of 5×10^{-3} M Tris, *p*H 7.0. The H_2M_2 form again appeared as the first lactic dehydrogenase activity recovered. The active fractions were combined and dialyzed overnight against saturated ammonium sulfate. The precipitate was centrifuged out, dissolved in 5×10^{-3} M Tris, *p*H 7.0, and dialyzed against the same buffer.

Step 10. Crystallization of the H_2M_2 Hybrid.—Ammonium sulfate was added slowly to the dialyzed solution from step 9 over a period of 3 to 5 hours until 40% saturation was obtained. Crystallization began as 40% saturation was approached. The crystals were harvested and recrystallized several times with ammonium sulfate in the same manner.

Preparation of HM_3 Hybrid from Chicken Leg.—Step 1. Crude Extract.—The muscles from whole chicken leg contain about 75% M subunits and 25% H subunits. Electrophoretic analyses on starch gel of crude leg muscle lactic dehydrogenase show substantial amounts of the HM_3 hybrid, along with an abundance of the expected M_4. However the M_4 is quite insoluble as compared with H_4, and can be extracted completely only in the presence of salt. In the isolation of HM_3 from chicken leg advantage was taken of this fact by using distilled water for the initial extraction, thus leaving most of the M_4 in the residue. While only about one-third of the lactic dehydrogenase present was extracted from the muscle mince with distilled water, there was a selective elimination of M_4 and thus an important initial purification.

The muscle tissue from 30 lb of fresh, skinned chicken legs was excised and twice ground in a meat grinder at 4°. The muscle mince was extracted with 15 liters of cold distilled water overnight. The extract was strained through cheesecloth and the filtrate was freed of precipitate by centrifugation at 4° and 35,000 × *g* in a Sharples continuous supercentrifuge. Approximately 960 mg of lactic dehydrogenase was extracted by this procedure. This quantity represents about one-third of the amount present in whole chicken-leg muscle. The analog ratio of the crude extract using deamino-DPNH with low pyruvate concentration and DPNH with high pyruvate concentration (Kaplan and Ciotti, 1961) gave a value of 2.0.

Step 2. Calcium Phosphate—Gel Adsorption.—Calcium phosphate–gel was prepared as described by Meister (1952) and washed free of chloride in nine 14-liter portions of distilled water. The washed gel was added stepwise to the centrifuged extract until all enzymatic activity was adsorbed. The gel was allowed to settle overnight, and the inactive supernatant was siphoned off and discarded. The gel was centrifuged at 900 × *g* and 4°, and the inactive supernatant was again discarded.

The gel was suspended in 10 liters of 0.15 M phosphate buffer, *p*H 7.5, at 4°. The gel was allowed to settle, the active supernatant was withdrawn, and the gel was centrifuged as above. The combined supernatants contained about 600 mg of enzyme. The precipitated gel was again washed with 10 liters of phosphate buffer and yielded another 300 mg of enzyme. The combined phosphate washings thus accounted for approximately 900 mg of dehydrogenase.

Starch-gel electrophoresis showed little selective adsorption of particular hybrids by the calcium phosphate gel, since the four hybrids originally present appeared again and with the same relative intensities of staining. The analog ratio was about 2.0.

Step 3. First Ammonium Sulfate Fractionation.—The combined phosphate washings from step 2 were brought to 45% saturation with ammonium sulfate, the *p*H being maintained at 7.0 by stepwise addition

of dilute ammonium hydroxide. The mixture was allowed to stand for 24 hours at 4° and was then filtered. Determination of activity of the filtrate showed 410 mg of lactic dehydrogenase. The precipitate was washed with two 2.5-liter portions of 45% saturated ammonium sulfate at 4°, followed by filtration. The first and second washings yielded, respectively, 85 and 30 mg of lactic dehydrogenase. The filtrate and washings combined thus accounted for 525 mg of lactic dehydrogenase, whereas the washed precipitate had virtually no lactic dehydrogenase activity.

Starch-gel electrophoresis of the active filtrate again showed the presence of four lactic dehydrogenase forms, with an apparent decrease in the intensity of staining of the most anodically migrating hybrid (presumably the pure heart-type). At this point, an analog ratio of 1.7 was obtained for the preparation.

Step 4. Second Ammonium Sulfate Fractionation.—The filtrate from the 45% ammonium sulfate step, comprising 27 liters, was brought to 60% saturation with ammonium sulfate; the *p*H was again maintained at 7.0 with dilute ammonium hydroxide. The mixture was allowed to stand overnight at 4°, then filtered, and the filtrate was assayed. Activity of the filtrate accounted for 66 mg of lactic dehydrogenase. Both the analog ratio of 1.3 and the starch-gel electrophoresis showed this filtrate to be predominantly M_4- and HM_3-type lactic dehydrogenases. Since only 66 mg of enzyme was accounted for by the 60% ammonium sulfate filtrate, the preparation was continued on the precipitate from the 60% ammonium sulfate step.

The precipitate was suspended in 500 ml of water and dialyzed against 20 liters of water at 4°, followed by dialysis against 5×10^{-3} M Tris buffer, *p*H 7.0, for 20 hours. Activities of the suspension before and after dialysis showed 230 mg of lactic dehydrogenase. Both analog ratio (2.0) and starch-gel electrophoresis showed that this preparation contained practically the same relative amount of all four hybrids as were present in the original crude extract.

Step 5. Carboxymethyl-Cellulose Chromatography.—A carboxymethyl-cellulose column was prepared as described by Pesce *et al.* (1964). The precipitate from the 60% ammonium sulfate step dissolved in water and dialyzed against 5×10^{-3} M Tris buffer, *p*H 7.0, as described in step 4, was added dropwise to the column. The protein mixture was eluted with 5×10^{-3} M Tris buffer, *p*H 7.0, and nine 500-ml fractions were collected. Both starch-gel electrophoresis and the analog ratios showed that the lactic dehydrogenase eluted from the column was almost exclusively H_4.

The enzyme still adsorbed to the column was collected in 12-ml aliquots by gradient elution with 2 liters of 5×10^{-3} M Tris buffer, *p*H 7.0, to which was gradually added from above a buffer of 2 liters of 5×10^{-3} M Tris, *p*H 7.0–0.2 M NaCl. Tubes 10–200 were eluted as a single enzyme fraction with an analog ratio of 1.4. Starch-gel electrophoresis showed that this enzyme is composed of the three slowest anodically migrating species (M_4, HM_3, and H_2M_2), and is totally free of H_3M and H_4. The combined 1200-ml volume accounted for 120 mg of lactic dehydrogenase. At this point the total protein content of the preparation as estimated by the relative optical densities at 280 and 260 mμ (Warburg and Christian 1941) was approximately 8 g.

Step 6. DEAE-Cellulose Fractionation at *p*H 7.6.—DEAE-cellulose was washed as described by Pesce *et al.* (1964), equilibrated in 0.5 M Tris buffer, *p*H 7.6, and washed with 5×10^{-3} M Tris buffer, *p*H 7.6. A column 3 × 40 cm was prepared.

The 120 mg of muscle and hybrid-type lactic dehydrogenases obtained in step 5 by gradient elution was precipitated by addition of ammonium sulfate to 70% saturation. The suspension was centrifuged and the precipitate was dissolved in 100 ml of cold distilled water. After dialysis against two 12-liter portions of 5×10^{-3} M Tris buffer, *p*H 7.6, the enzyme was added to the DEAE-cellulose column.

The mixture was eluted with 5×10^{-3} M Tris buffer, *p*H 7.6. The initial 250 ml of eluate showed no activity. The next 500 ml of eluate was collected in three fractions, the first showing pure M_4 lactic dehydrogenase on starch gel, the succeeding two fractions showing a gradually increasing amount of the slowest anodically moving hybrid (HM_3) in addition to M_4. Analog ratios of these three fractions were about 1.5. These three active fractions accounted for approximately 25 mg of lactic dehydrogenase, presumably leaving about 90 mg still adsorbed to the column.

The enzyme still adsorbed to the column was eluted with a buffer gradient of 5×10^{-3} M Tris buffer, *p*H 7.6 (1 liter) as the main reservoir to which was added dropwise from above a buffer of 5×10^{-3} M Tris, *p*H 7.6, NaCl 10^{-1} M. Fractions, 20 ml each, were collected and assayed for lactic dehydrogenase content. Tubes 1–20 showed no activity, whereas lactic dehydrogenase activity corresponding approximately to 70 mg of enzyme was eluted in a single peak from tubes 23–39. Starch-gel electrophoresis of tube 22 showed the presence of predominantly HM_3 hybrid with some contamination by M_4. Tube 28 showed almost solely HM_3 with a trace of a faster moving hybrid. Analog ratios of earlier and later tubes in the enzyme peak gave values of 1.4.

Tubes 24–31 were pooled and subjected to further purification of the almost pure HM_3 hybrid. At this point in the preparation, the 170-ml fraction of relatively pure HM_3 consisted of 50 mg of enzyme in a total protein content of 168 mg. The enzyme was crystallized by the addition of ammonium sulfate to 70% saturation.

Step 7. DEAE-Cellulose Fractionation at *p*H 7.0.—A column of DEAE-cellulose, 2.5 × 30 cm, was prepared as in step 6 and equilibrated with 1×10^{-3} M Tris buffer, *p*H 7.0. The crystallized HM_3 fraction from step 6 was dissolved in 10 ml of cold water, dialyzed against 6 liters of 1×10^{-3} M Tris buffer, *p*H 7.0, and added to the column. The enzyme was eluted with 1×10^{-3} M Tris buffer, *p*H 7.0 and collected in 10-ml fractions. Virtually the entire enzymatic activity was concentrated in tubes 13 and 14, with another 10% located in tubes 12 and 15.

Starch-gel electrophoresis showed that the enzyme in tubes 12 and 14 with a lactic dehydrogenase free of other hybrids, while a second hybrid began to appear in tube 16. Consequently, tubes 12–15 inclusive were pooled as pure HM_3 hybrid and crystallized by addition of ammonium sulfate to 65% saturation. The crystalline enzyme was centrifuged and twice washed with 15 ml of 65% ammonium sulfate at 4°. Both supernatant and washings showed virtually no lactic dehydrogenase activity. Assay of the crystalline enzyme showed that it was 48 mg of lactic dehydrogenase. The crystalline enzyme so prepared was then characterized by ultracentrifugal properties, Tiselius electrophoresis, complement fixation, amino acid analysis, and fingerprinting. Starch-gel electrophoresis showed this final product to be a single electrophoretic band corresponding to the HM_3 hybrid.

The purity of the preparation was supported by the presence of a single sedimenting boundary in the ultracentrifuge and by a single migrating boundary in Tiselius electrophoresis.

Digestion with Trypsin.—A typical tryptic digestion was carried out with Worthington twice-crystallized, salt-free trypsin dissolved in 10^{-3} M hydrochloric acid as 0.5% w/v. The lactic dehydrogenase to be digested was dialyzed against 10^{-1} M ammonium bicarbonate, heated to 95° for 5 minutes, then cooled, and the coagulated protein was dispersed. One per cent protein weight of trypsin was added, followed by a drop of toluene to prevent bacterial growth. The digestion mixture was incubated at 37° in a shaker for 48 hours until almost all of the protein had gone into solution. The digest was spotted on Parafilm in volumes calculated to leave a residue of 1 or 2 mg after evaporation.

Fingerprinting of Tryptic Digests.—The fingerprinting method of Ingram (1959), as modified by Baglioni (1961), was employed. Electrophoresis was carried out with a pyridine–acetic acid–water buffer (100:4:900), *p*H 6.4. A potential of 25 v/cm was applied for 3.5 hours along the bottom of a sheet 45 cm long. The second dimension (25 cm) was developed by ascending chromatography with a solvent of 1:1 pyridine–isoamyl alcohol saturated with water. After drying, the fingerprints were stained by dipping through 0.2% (w/v) of ninhydrin in 100:1 acetone-pyridine. Ehrlich's stain for tryptophane (Smith, 1960) was effectively applied directly to ninhydrin-treated fingerprints. Staining for histidine with *p*-anisidine–isoamyl nitrite and for arginine with the Sakaguchi reagent (Smith, 1960) was carried out after first bleaching the ninhydrin color with 0.1 N HCl in acetone.

Amino Acid Analyses.—The amino acid compositions of the various lactic dehydrogenases were determined by the procedure reported in paper I of this series (Pesce *et al.*, 1964). The amino acid values for M_4 and for chicken heart H_4 are those given in that same study. For HM_3, H_2M_2, and chicken liver H_4, the amino acid data were obtained from one preparation of each enzyme. Duplicate amino acid determinations were made of chicken liver H_4, only one of HM_3, and four of H_2M_2.

Molecular Weight Determinations.—The molecular weights of these enzymes were determined by the Ehrenberg procedure, as described in another publication (Pesce *et al.*, 1964). Only one determination of each of the enzymes was possible. The variation in the molecular weights in the procedure employed by Pesce *et al.* was ± 7,000 g, and the same precision may be expected with the results reported here.

Catalytic and Immunological Studies.—Determinations of enzyme activity and studies of analog ratios were carried out as outlined elsewhere (Pesce *et al.*, 1964; Kaplan and Ciotti, 1961). Complement-fixation studies were performed according to the method of Wasserman and Levine (1961). For studies on enzyme inhibition by antibody, an equal amount of serum was added in all cases to the same amount of enzyme. After remaining for 1 hour at 25° the reaction mixture was assayed for residual enzyme activity.

Results

Fingerprint Patterns.—Figure 1 shows the peptide patterns and specific staining obtained for H_4, M_4, 1:1 mixed H_4 and M_4, H_2M_2, and HM_3. The patterns have been overlaid with a grid to facilitate identification of specifically stained peptides with peptides in the overall ninhydrin-stained pattern of each particular enzyme. The grids thus are for reference across the page within a single lactic dehydrogenase form and are to be disregarded when one compares one enzyme form to another.

Comparison of the patterns obtained for H_2M_2 with those for a 1:1 mixture of H_4 and M_4 shows virtual identity. In the ninhydrin-stained fingerprints one point of nonidentity occurs in the region of 80–40 (x, y) of the 1:1 mixture which shows three peptides, whereas the corresponding area in the H_2M_2 pattern (70–40) has only two peptides with a "new" peptide appearing at 90–38. The double peptide around point 15-5 in the 1:1 mixture corresponds to the double peptide around point 3–5 in the H_2M_2 pattern which failed to show a peptide corresponding to the one at 0-0 in the 1:1 mixture. Comparison of the arginine-stained fingerprints for the 1:1 mixture and for the H_2M_2 hybrid again shows almost complete identity except for the appearance of two peptides in the H_2M_2 pattern at points 70-18 and 70-40. There also appears to be an upward shift in the spot originally at 60-10 in the mixture which may correspond to the spot at 50–20 in the hybrid. The histidine and tryptophan patterns of the mixture and the hybrid show total identity.

The patterns obtained for HM_3, when compared to those for the 1:1 mixture of H_4 and M_4, show strong similarities. In the pattern for tryptophan and in that of histidine, HM_3 closely resembles the H_4-M_4 mixture, whereas the arginine pattern approximates more the pattern for M_4 than for the mixture.

Amino Acid Analyses.—Table I compares the amino acid composition of the H_4 enzyme isolated from both heart and liver, the M_4 from breast muscle, and the H_2M_2 from liver. The H_4 isolated from heart and liver are closely similar in their compositions. The amino acid content of H_2M_2 agrees fairly well with what might be predicted from the compositions of H_4 and M_4. This is particularly striking with the histidine analyses. There is considerable destruction of serine and threonine between the 24- and 48-hour acid hydrolysates of H_2M_2 and the values for these are lower than expected for an enzyme which is intermediate between the two extremes.

Only one amino acid analysis was carried out on the HM_3 hybrid. The histidine value of this hybrid

TABLE I

AMINO ACID COMPOSITIONS OF LACTIC DEHYDROGENASES FROM CHICKEN

	H_4 from Heart	H_4 from Liver	H_2M_2 from Liver	M_4 from Breast Muscle
Lys	99	102	106	112
His	30	30	45	63
Arg	35	32	35	35
Asp	129	122	122	125
Thr	75	75	63	51
Ser	107	102	100	110
Glu	122	115	108	102
Pro	38	42	42	44
Gly	96	96	101	104
Ala	88	84	82	81
Val	125	135	122	121
Met	25	23	27	31
Ileu	66	73	83	85
Leu	149	146	137	121
Tyr	31	30	23	19
Phe	19	19	25	27

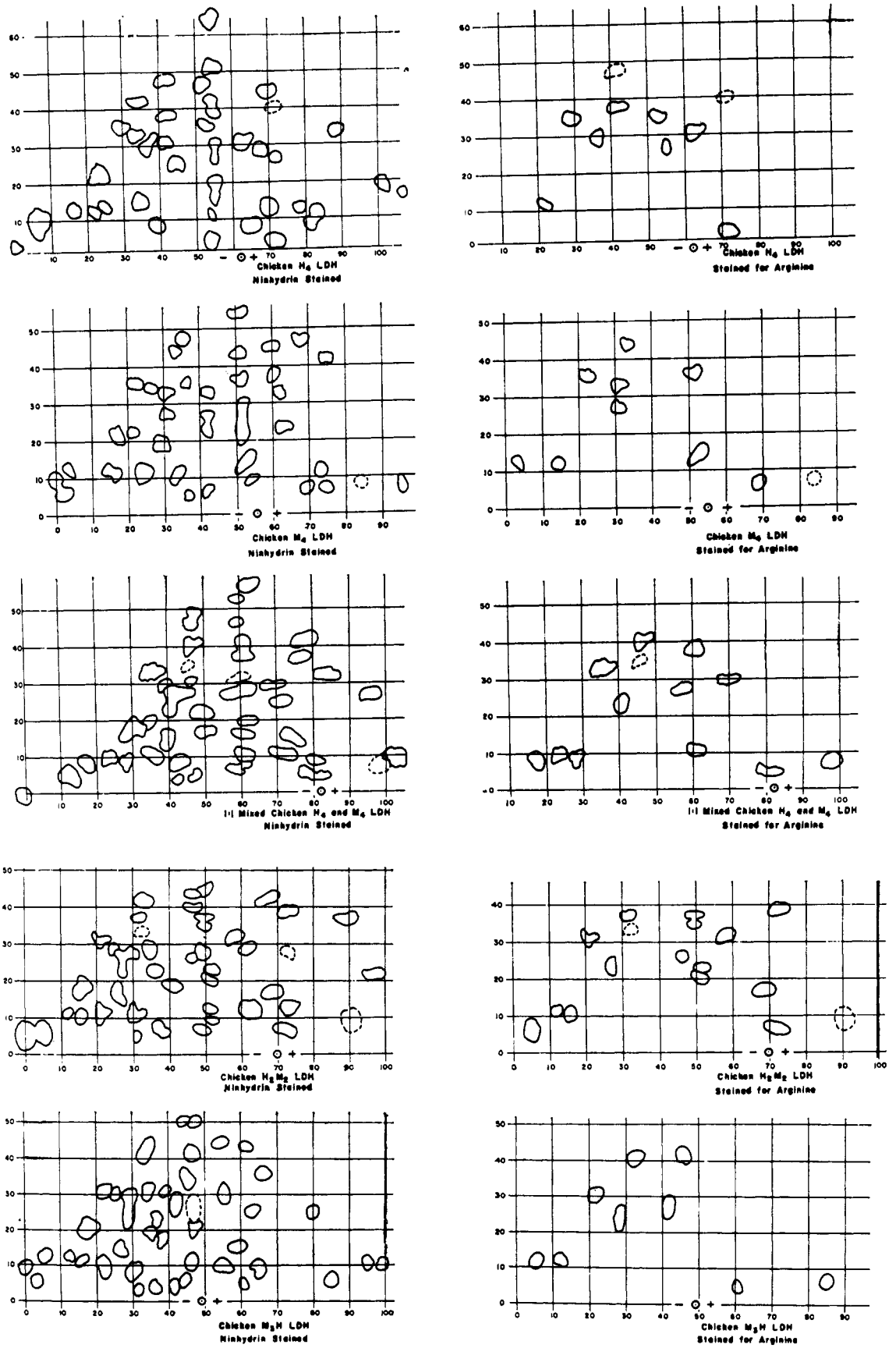
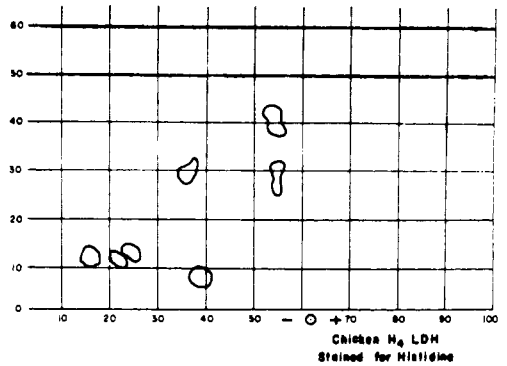
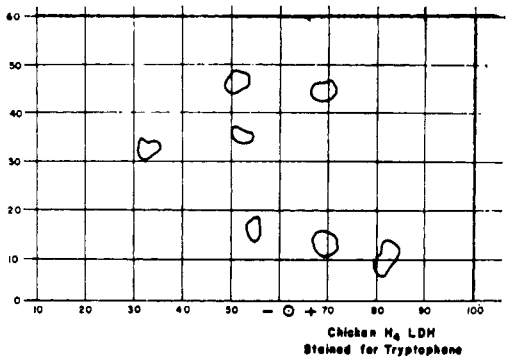
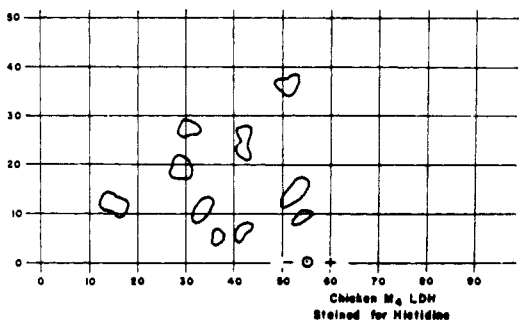
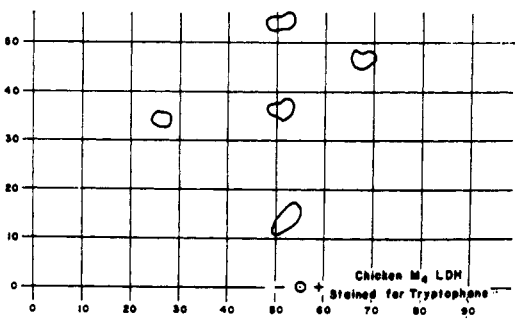


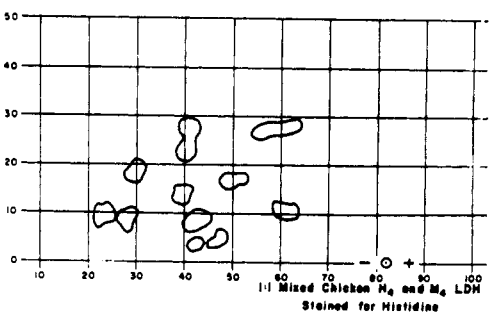
FIG. 1.—Fingerprint patterns and specific staining of tryptic digests of H_4, M_4, 1:1 mixed H_4 and M_4, H_2M_2, and HM_3 lactic dehydrogenases from chicken. Grid coordinates are for correlation of ninhydrin-stained peptides with those stained

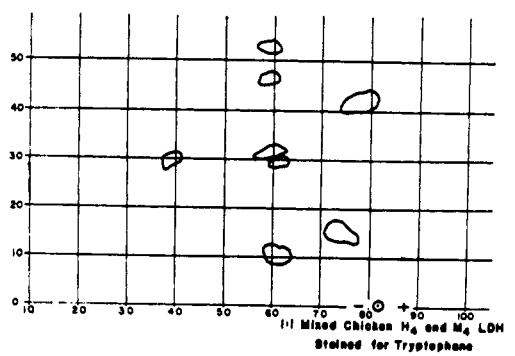


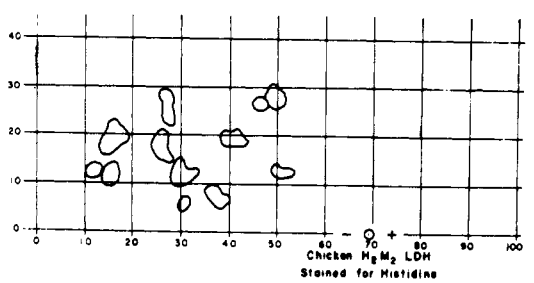


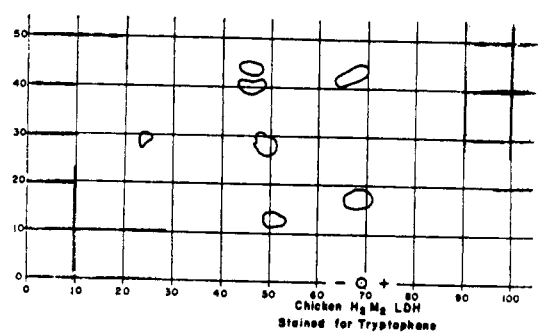


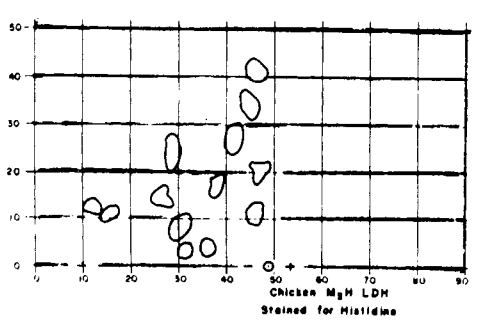


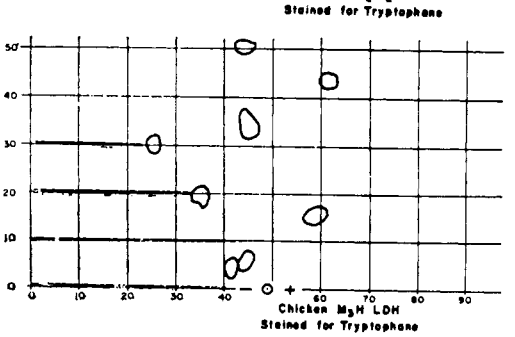












specifically for arginine, histidine, or tryptophan only within a given lactic dehydrogenase type.

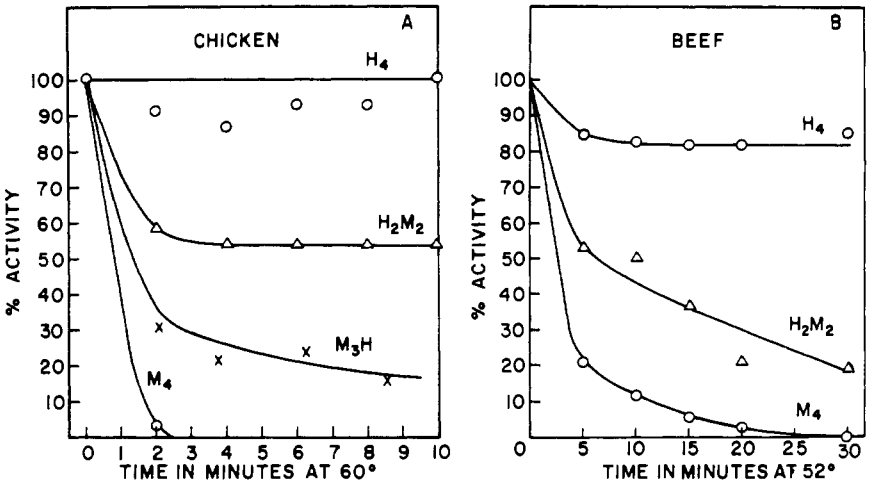


FIG. 2.—Heat stabilities of crystalline lactic dehydrogenases from chicken and of beef lactic dehydrogenases removed from starch grain. The activities of aliquots at various intervals were measured with 3×10^{-4} M pyruvate and 1×10^{-4} M DPNH.

was in excellent agreement with the predicted value; most of the other amino acids were only in fair agreement with the expected values.

Molecular Weights.—Ultracentrifugal studies showed the molecular weight of H_4 isolated from chicken liver to be 154,000 g, which compares favorably to the value of 151,000 g reported for the enzyme isolated from chicken heart (Pesce *et al.*, 1964). The molecular weights of the H_2M_2 and HM_3 enzymes were, respectively, 154,000 and 146,000. None of these determinations varied significantly from the range of values already established for the M_4 and H_4 enzymes.

The Svedberg constant for the HM_3 enzyme was 7.21 $s^\circ_{20,w}$ and the concentration dependence was 0.72 $s^\circ_{20,w}$ per mg/ml. These values are somewhat higher, but probably not significantly different, from those found for the H_4 and M_4 proteins.

Heat Stabilities.—Figure 2A compares the heat stabilities of the crystalline H_4, M_4, and H_2M_2 preparations. The H_4 was quite stable at 60°, whereas the M_4 was very labile, being completely inactivated in 2 minutes at this temperature. It was of interest to note that with H_2M_2 about 50% of activity was lost in a few minutes and then there was no further loss of catalytic activity. With HM_3 there was roughly a 75% decrease in enzymatic activity, after which a slower rate of inactivation occurred. The data in Figure 2A suggest that the H units of the H_2M_2 could retain their activity even though the M units were denatured. The H unit in the HM_3 hybrid seemed to be somewhat more labile than the H units of H_2M_2.

Figure 2B shows the heat labilities of some of the beef lactic dehydrogenase forms.[2] The beef H_4 was somewhat more sensitive to heat than the corresponding chicken enzyme. It is of interest that the beef H_2M_2 showed kinetics of inactivation somewhat similar to the chicken H_2M_2. However, it is evident from the curves that the H units of the chicken H_2M_2 were relatively more stable to temperature than the H unit of the beef hybrid. Comparative data may also indicate that the forces between the "native" H units and denatured M units are firmer in the chicken than in the beef system. After 50% inactivation of the chicken H_2M_2, little change in the electrophoretic migration occurred as compared to the normal movement of the hybrid.

[2] The beef lactic dehydrogenase forms were obtained after electrophoresis on starch grain.

Table II presents data indicating that the hybrids were somewhat more unstable than the pure forms when stored in a Tris buffer at 4° for a number of days. The beef hybrids appeared to be considerably more labile than the chicken hybrids. It is of interest that the beef H_3M was more stable than the HM_3 hybrid units.

Immunological Properties.—Table III shows that antibodies to the H_4 and M_4 enzymes inhibited the enzymatic activity of the homologous enzyme but not the other enzymes. The hybrids were inhibited by both antibodies in accordance with the amount of H and M subunits.

TABLE II

INACTIVATION OF VARIOUS FORMS OF LACTIC DEHYDROGENASE FROM BEEF AND CHICKEN AFTER STANDING IN TRIS BUFFER (*p*H 7.5, 0.1 M)

Enzyme Form	Activity Remaining after 15 Days at 4°	
	Beef[a] (%)	Chicken[b] (%)
H_4	85	95
H_3M	71	100[c]
H_2M_2	36	68
HM_3	21	54
M_4	82	83

[a] The beef enzymes were all removed from starch grain after electrophoresis. [b] All the chicken enzymes were crystalline preparations except H_3M. [c] This preparation contained approximately 10% of the H_4 form.

TABLE III

CATALYTIC AND IMMUNOLOGICAL PROPERTIES OF CRYSTALLINE CHICKEN LACTIC DEHYDROGENASES

Enzyme Form	Inhibition		DeDPNH$_L$/ DPNH$_H$[a]	Inhibition by 3×10^{-4} M Oxalate (%)
	By Anti-H_4 Antibody (%)	By Anti-M_4 Antibody (%)		
H_4	100	0	3.2	84
H_2M_2	46	51	1.97	61
HM_3	18	80	1.40	46
M_4	0	100	0.55	33

[a] Ratio of rates of reaction of DeDPNH (hypoxanthine analog of reduced DPN) at pyruvate concentration of 3×10^{-4} M and DPNH at a pyruvate concentration of 1×10^{-2} M (Kaplan and Ciotti, 1961).

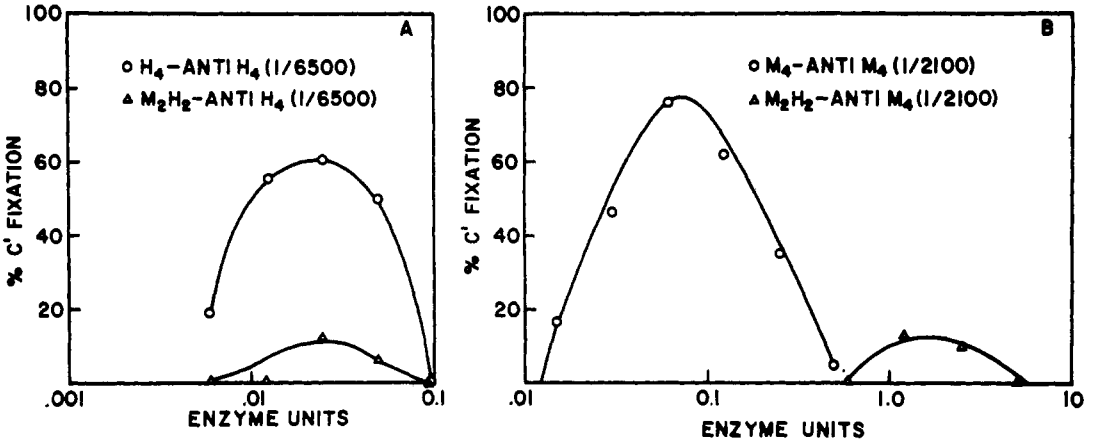


FIG. 3.—Determination of cross reaction between crystalline H_2M_2 from chicken with rabbit anti-chicken H_4 and rabbit antichicken M_4. The complement fixation technique of Wasserman and Levine (1961) was used.

Figure 3 shows the quantitative complement fixation curves of the H_2M_2 form with the antibodies to the M_4 and H_4 forms. It is evident that the hybrid reacted more poorly with both antibodies as compared with the respective pure types. The reaction of the H_2M_2 to the two antibodies was somewhat different. Much more of the M_4 antibody was required to give peak fixation with the same hybrid.

In a reaction with HM_3 using antibody to M_4, it was observed that twice the concentration of antibody was needed for reaction with HM_3 as compared to reaction with M_4. At the antibody dilutions employed, no reaction was observed between HM_3 and anti-H_4 by complement fixation.

Antibody prepared in rabbits to chicken HM_3 produced a somewhat anomalous result, reacting rather better with chicken M_4 than with HM_3 itself. Reaction of anti-HM_3 with M_4, HM_3, and H_4 was observed on Ouchterlony plates, and with an antibody concentration five times greater than that required for the M_4–anti-HM_3 interaction, reaction between H_4 and anti-HM_3 was demonstrated with complement fixation.

Catalytic Characteristics.—Table III also presents data illustrating that the crystalline chicken hybrids have analog ratios intermediate between those for the pure types. Oxalate was observed to inhibit the chicken H_4 enzyme more strongly than the M_4 form. The inhibition of the two crystalline hybrids by oxalate was again intermediate between the two pure forms.

Discussion

The evidence presented here supports the view that the lactic dehydrogenase forms which migrate on starch-gel electrophoresis to points intermediate between the two extreme types are indeed hybrids of the two extreme forms. The fingerprint patterns and specific staining show that crystalline H_2M_2 from chicken liver produces the same pattern on tryptic digestion as does a 1:1 mixture of crystalline H_4 from chicken heart with crystalline M_4 from chicken breast muscle. Similarly, the patterns obtained with crystalline HM_3 from chicken leg muscle are almost identical to those obtained from the 1:1 mixture with some few exceptions emphasizing its closer relationship to M_4 than H_4.

The fingerprint patterns presented in Figure 1 also indicate that the H_4 and M_4 lactic dehydrogenases may have many points of divergence in their primary sequences. This may be the case since both the H_4 and M_4 patterns show approximately 40 peptides, while the 1:1 mixture of the two shows more than fifty distinct spots. Thus there may be about thirty peptides common to both H_4 and M_4, and perhaps ten peptides characteristic only of H_4, and ten others unique to M_4.

Amino acid analysis of the crystalline H_2M_2 hybrid from chicken again indicates the hybrid nature of this lactic dehydrogenase form. This is particularly striking in the values for histidine, which showed the greatest variation among the amino acids in H_4 and M_4. The histidine content of crystalline H_2M_2 and of HM_3 formed a neat progression in values running from H_4 to M_4.

The amino acid data also establishes that there is close agreement between the amino acid composition of H_4 isolated from chicken heart and H_4 from chicken liver.

The molecular weights observed for the crystalline hybrids from chicken were in the range of values obtained earlier (Pesce *et al.*, 1964) for crystalline H_4 and M_4.

The heat stability of the pure and hybrid forms of lactic dehydrogenase from both chicken and beef again demonstrates the intermediate behavior of the hybrids. Of particular interest is the observation that the H subunits of the chicken hybrids appear to retain activity even after the M subunits have lost activity. Thus the heat stability curve for chicken H_2M_2 in Figure 2A leveled off at 50% activity, while that for HM_3 did so with about 25% activity remaining. This observation is less clear-cut using beef hybrids isolated from starch grain.

The data presented in Table II on the loss of activity with time show that the hybrids are more unstable on storage than are the pure forms. The fact that beef H_3M is more stable than HM_3 is to be expected because of the greater tendency toward denaturation of the M units.

The catalytic and immunochemical data presented in Table III also show a progression of values running from H_4 to M_4 and again establishing the hybrid nature of the intermediate forms. We believe that the data presented in this paper strongly support the previously reported concept (Cahn *et al.*, 1962) of the nature of the multiple electrophoretic forms of lactic dehydrogenase.

ACKNOWLEDGMENTS

We are grateful to Messrs. Louis Costello and Fred Castillo, and to Mrs. Shirley White for their assistance in various aspects of this work.

REFERENCES

Appella, E., and Markert, C. L. (1961), *Biochem. Biophys. Res. Commun. 6*, 171.
Baglioni, C. (1961), *Biochim. Biophys. Acta 48*, 392.
Cahn, R. D., Kaplan, N. O., Levine, L., and Zwilling, E. (1962), *Science 136*, 962.
Di Sabato, G., and Kaplan, N. O. (1964), *J. Biol. Chem. 239*, 438.
Fine, I. H., and Costello, L. (1963), *Methods Enzymol. 6*, 958.
Fine, I. H., Kaplan, N. O., and Kuftinec, D. (1963), *Bio-Chemistry 2*, 116.
Ingram, V. M. (1959), *Biochim. Biophys. Acta 28*, 539.
Kaplan, N. O., and Cahn, R. D. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 2123.
Kaplan, N. O., and Ciotti, M. M. (1961), *Ann. N. Y. Acad. Sci. 94*, 701.
Markert, C. L. (1963), *Science 140*, 1329.
Markert, C. L., and Appella, E. (1963), *Ann. N. Y. Acad. Sci. 103*, 915.
Meister, A. (1952), *Biochem. Prepn. 2*, 18.
Pesce, A., McKay, R. H., Stolzenbach, F., Cahn, R. D., and Kaplan, N. O. (1964), *J. Biol. Chem. 239* (in press).
Smith, I. (1960), Chromatographic and Electrophoretic Techniques, Vol. I, New York, Interscience, pp. 96–97.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Wasserman, E., and Levine, L. (1961), *J. Immunol. 87*, 290.

An Optical Rotatory Dispersion Study of Aspartic Amino Transferase*

PAOLO FASELLA† AND GORDON G. HAMMES

From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge

Received October 23, 1963

An optical rotatory dispersion study of several forms of the enzyme aspartic amino transferase has been carried out in the wavelength range of 300–600 mμ. The pyridoxal and pyridoxamine enzymes in aqueous solution display anomalous rotatory dispersion indicating a strong interaction between coenzyme and enzyme. When ketoglutarate is added to the aldimine enzyme to form a complex, the rotatory dispersion curve is unchanged. On the other hand, addition of hydroxylamine, yielding an oxime, causes complete disappearance of the Cotton effects. The apoenzyme and the denatured enzyme also have plain dispersion curves. The b_0 for the oxime enzyme is quite high (−490°) suggesting a highly ordered structure, whereas the apo- and native enzymes have quite low b_0 values (> −100°). However, the a_0 values for the oxime and apoenzyme are essentially identical (∼ −220°), while that for the enzyme in urea is markedly changed (−490°). According to current theories, this seems to indicate that pyridoxal phosphate has a profound ordering effect on the enzyme structure.

The use of optical rotatory dispersion for providing information about the conformation of proteins and about their interaction with prosthetic groups is now well established (Urnes and Doty, 1961). The enzyme aspartic amino transferase can exist in several distinct forms (Jenkins and Sizer, 1960; Lis *et al.*, 1960). At high *p*H values (>8) an active enzyme containing tightly bound pyridoxal phosphate and having an absorption maximum at 362 mμ is formed. At lower *p*H values (<5) the enzyme is inactive and its spectral maximum shifts to 430 mμ. In both cases the aldehyde group of the pyridoxal phosphate forms an internal Schiff base with an ε-amino group of the protein (Turano *et al.*, 1961; Hughes *et al.*, 1962). These two forms are jointly termed the *aldimine enzyme*. The active aldimine enzyme can react with amino acid substrates to give keto acids and a protein-containing pyridoxamine phosphate with an absorption maximum at 333 mμ (the aminic enzyme) (Jenkins and Sizer, 1960; Lis *et al.*, 1960). The inactive apoenzyme can be prepared by complete removal of the pyridoxal phosphate. (Banks and Vernon, 1961; Wada and Snell, 1962). The aldimine enzyme can also react with dicarboxylic acids and carbonyl reagents, which are competitive inhibitors of the enzyme reaction, to form inactive complexes (Polyanovsky and Torchinsky, 1961; Velick and Vavra, 1962b; Jenkins and Sizer, 1963; Banks *et al.*, 1963; Karpeisky *et al.*, 1963; Hammes and Fasella, 1964).

An investigation of the optical rotatory dispersion of the various forms of the enzyme is of interest to obtain further information about the apoprotein-coenzyme and enzyme–quasi substrate interactions and to detect possible conformation changes occurring in the protein moiety of the enzyme when it reacts with coenzyme, substrate, and substrate analogs.

EXPERIMENTAL

Aspartic amino transferase from pig hearts was prepared according to Lis (1958). This preparation is homogeneous in the ultracentrifuge and in zonal and free-phase electrophoresis (Passalacqua, 1961). The pyridoxamine form of the enzyme was prepared according to Lis *et al.* (1960). The apoenzyme was prepared by the procedure described by Wada and Snell (1962) for the resolution of transaminases. The apoenzyme thereby obtained had less than 5% of the original activity. The activity could be restored (more than 90% of the original values) after 30 minutes' incubation with 10^{-4} M pyridoxal phosphate at *p*H 8.05 in 0.05 M potassium phosphate buffer at 20°. The denatured enzyme was prepared by putting the pyridoxal enzyme into a buffered solution saturated with recrystallized urea at 20° (9 M). This solution

* This work was supported by a grant (MG-07803) from the National Institutes of Health, U. S. Public Health Service.

† Permanent address: Istituto di Chimica Biologica, University of Rome, Rome, Italy