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Purification and Properties of Chicken Heart Mitochondrial and Supernatant Malic Dehydrogenases*

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ABSTRACT: The mitochondrial and supernatant forms of malic dehydrogenase from chicken heart have been isolated and crystallized. Both enzymes are homogeneous by ultracentrifugal analysis, have a molecular weight of approximately 67,000, and from a number of criteria appear to be dimeric in structure. The supernatant and mitochondrial enzymes differ markedly in their amino acid composition and in their peptide maps.

The two enzymes may also be readily differentiated on the basis of their differing sensitivities to substrate inhibition by oxaloacetate and L-malate, and by their reactivity with coenzyme analogs. The mitochondrial enzyme is more sensitive to heat inactivation than is the supernatant enzyme. Like the pig and horse heart mitochondrial malic dehydrogenases, the chicken mitochondrial enzyme is devoid of tryptophan. Rabbit antisera directed against chicken mitochondrial malic dehydrogenase inhibit this enzyme and show a strong reaction by double-diffusion and complement fixation procedures. These antisera show no detectable inhibition or cross-reaction with the supernatant enzyme. The reverse is true of antisera to the supernatant enzyme which show no cross-reaction with the mitochondrial enzyme. Fluroescence titrations of the two enzymes with the reduced acetylpyridine analog of diphosphopyridine nucleotide indicate that 2 moles of coenzyme is bound/mole of enzyme. The fluorescence characteristics of the two enzymes are compared.

In recent years there have been a number of reports that there are two major separable forms of malic dehydrogenase present in the tissues of most animals (Delbrück *et al.*, 1959a,b; Wieland *et al.*, 1959; Thorne, 1960; Englard and Breiger, 1962; Siegel and Englard, 1961). One form of the enzyme is found in the mitochondria, while the other form is present in the supernatant fraction. These two types have been found to possess different catalytic, physical, and immunological properties and appear to be under the control of separate genes. As part of a continuing comparative study on the properties of dehydrogenases, the present communication reports the purification and properties of the mitochondrial and supernatant malic dehydrogenases of chicken heart.

Materials and Methods

Materials. Pig mitochondrial malic dehydrogenase was purchased from C. F. Boehringer and Soehne, through Calbiochem. Since the commercial preparation was impure, as shown by ultracentrifugal analysis, it was further purified by chromatography on carboxymethylcellulose and Sephadex G-100 and was crystallized by addition of ammonium sulfate. Pig supernatant malic dehydrogenase was purchased from General Biochemicals and was further purified by chromatography on DEAE-cellulose and Sephadex G-100. Crystalline tuna mitochondrial malic dehydrogenase and ostrich supernatant malic dehydrogenase were prepared in this laboratory by methods to be described elsewhere.

Pyridine nucleotide coenzymes and coenzyme analogs were purchased from P-L Biochemicals, Inc. The acetylpyridine analog[1] (APDPN) was reduced by the method described by Rafter and Colowick (1957) for the enzymic reduction of DPN^+. The reduced analog was stored as the barium salt; before use, the ratio of absorbancy at 260:363 mμ was checked; the coenzyme was used only when this ratio was below 1.75. Concentrations of the reduced coenzymes were calculated from their absorbancies (ϵ 6.22×10^3 at 340 mμ for DPNH; ϵ 9.1×10^3 at 363 mμ for APDPNH). Oxaloacetic acid, L-malic acid, and D-malic acid were purchased from Nutritional Biochemicals Corp., and β-mercaptoethanol was purchased from the Eastman Kodak Co. DEAE-cellulose and carboxymethylcellulose were obtained

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[1] Abbreviations used: APDPN, acetylpyridine analog; DPN^+, oxidized diphosphopyridine nucleotide; DPNH, reduced DPN^+; TPN^+, oxidized triphosphopyridine nucleotide; TPNH, reduced TPN^+.

from the Brown Co., and Sephadex G-100 came from Pharmacia Inc. All other chemicals were of reagent grade.

Determination of Catalytic Properties. Enzyme assays were made in a Zeiss spectrophotometer PMQ II at 25°. Enzymatic activity was determined by the procedures described by Thorne *et al.* (1963), except that in the case of oxidized coenzyme, or coenzyme analog, 0.1 M sodium pyrophosphate, pH 9.0, was used rather than 0.09 M sodium glycinate.

Determination of Protein Content. In the purification of the enzymes, protein was determined spectrophotometrically by measuring the absorption of light at wavelengths of 280 and 260 mμ, as described by Warburg and Christian (1941). For the crystalline enzymes the experimentally determined extinction coefficients were used.

Starch Gel Electrophoresis. Gel electrophoresis was carried out in 14% starch gels with a phosphate–citrate buffer, pH 7.0, at 10 v/cm, as described elsewhere (Fine and Costello, 1963). A specific tetrazolium staining mixture was used to locate malic dehydrogenase activity (Thorne *et al.*, 1963).

Amino Acid Analyses. The amino acid compositions of the malic dehydrogenases, with the exception of tryptophan, were obtained according to the procedure of Moore *et al.* (1958) using a Beckman-Spinco automatic amino acid analyzer. Hydrolysis times of 24 and 48 hr with constant-boiling HCl were used in the standard determinations. Cysteine residues were determined as cysteic acid following performic acid oxidation (Hirs, 1956).

Tryptophan Analyses. The tyrosine to tryptophan ratio was obtained according to the procedure of Bencze and Schmid (1957) and Goodwin and Morton (1946). These were converted into tryptophan content by assuming the tyrosine residue values obtained by the amino acid analyses to be correct. In the first procedure, a tangent was drawn to the absorption peaks at 290 and 282 mμ. The resulting slope was converted into a tyrosine to tryptophan ratio with the constants given by these authors. From the same spectra, the absorbance values at 280 and 294.4 mμ, corrected for extraneous absorption, were used to determine the ratio from the formula obtained by Goodwin and Morton.

Peptide Mapping. Tryptic digestion and peptide mapping of the tryptic digests were carried out by the procedures used by Fondy *et al.* (1964) for lactic dehydrogenases.

Fluorescence and Coenzyme Binding Studies. Fluorometric investigations were made in a Zeiss spectrofluorometer, Model ZFM4c. The fluorescence emission spectra were corrected for variations in the photomultiplier efficiency and monochromator dispersion at different wavelengths.

Molecular Weight Determinations. Ultracentrifugal analyses of the proteins were carried out in a Beckman-Spinco Model E ultracentrifuge equipped with a Philpot–Svensson optical system and with interference optics. Sedimentation constants and molecular weights were determined either by the Ehrenberg (1957) procedure, using the methods outlined by Pesce *et al.* (1964), or by the method of Yphantis (1964). Molecular weights were calculated from the ratio S/D, obtained by the Ehrenberg procedure, using the conventional formula (Lamm, 1929); partial specific volumes of 0.74 were calculated for both chicken malic dehydrogenases from the amino acid compositions, using values published for the component amino acids (Cohn and Edsall, 1943). Molecular weights were also calculated from the apparent diffusion constants (Ehrenberg, 1957) and the sedimentation constants. The sedimentation constants were obtained from sedimentation velocity experiments performed at the same protein concentration and temperature as the diffusion experiments.

Temperature Stabilities. The crystalline enzymes were diluted at least 1000-fold in complement fixation buffer (see composition below) and heated at various temperatures. Temperature was controlled to within ±0.5°. Samples were withdrawn at various times, chilled immediately, and assayed for malic dehydrogenase activity.

Immunological Procedures. Rabbit antisera directed against the crystalline chicken mitochondrial and supernatant malic dehydrogenases were prepared in the following manner. A normal saline solution (1 ml) containing 10 mg of crystalline enzyme was mixed with an equal volume of Freund's adjuvant and injected into the toepads and thigh muscles. First-course sera were obtained after about 3 weeks. The rabbits then received intravenous injections of 10 mg of enzyme (in solution), dispensed as four 2.5-mg portions during an 8-day period. Second-course sera were obtained 5–8 days after the last injection. The rabbits then received intraperitoneal injections of 10 mg of enzyme, following the above time schedule, and third-course sera were obtained 5–8 days after the last injection. If necessary, this last series of injections was repeated to obtain fourth-course sera. In the later stages of this work further antisera were prepared using the method described by Plescia *et al.* (1964), which involves the use of methylated bovine serum albumin and which requires much smaller amounts of enzyme. A similar time schedule was followed for the injection of antigen.

The use of immunological methods for the study of the structure of specific proteins requires that the immune systems employed be fully characterized. This was accomplished in the following manner for the two immune systems used in the present investigation. Double diffusion was performed by the method of Ouchterlony (1948) in 1% agar containing 0.9% NaCl and 0.2 mg/ml of powdered merthiolate, adjusted to pH 8.0. The antibody well contained undiluted antiserum. The antigen wells contained varying amounts of the enzyme used for eliciting the antibody, and one well contained a crude extract of chicken heart. The wells were 0.6 cm apart and diffusion was allowed to continue for at least 48 hr at 5°. At the end of the experiment the agar plates were washed briefly

with buffer and placed in a tetrazolium staining mixture specific for malic dehydrogenase (Thorne *et al.*, 1963) so as to ascertain whether the precipitin band and the band of malic dehydrogenase activity were coincident. All the antimitochondrial malic dehydrogenase and antisupernatant malic dehydrogenase antisera used in this study gave a single, sharp precipiting band when tested either against their homologous antigens, or with crude heart extract. These precipitin bands stained for malic dehydrogenase activity.

Quantitative microcomplement fixation was performed according to the procedure of Wasserman and Levine (1961) using the 7.0-ml total reaction volumes described in their footnote 3. All reagents were diluted in a buffer mixture containing 0.14 M NaCl; 0.01 M Tris, pH 7.5; 5×10^{-4} M $MgSO_4$; 1.5×10^{-4} M $CaCl_2$; and 0.1% bovine serum albumin; the final pH of the mixture being adjusted to 7.5. All antisera used in the complement fixation studies were tested over a wide range of antigen concentrations and showed only a single fixation peak.

Inhibition of malic dehydrogenase activity by antisera was measured as described by Wilson and Kaplan (1964) using the complement fixation buffer detailed above as diluent. Immunoelectrophoresis was performed as described by Grabar and Williams (1955).

Preparation of Mitochondrial and Supernatant Malic Dehydrogenases from Chicken Heart. Over the past 3 years we have made eight preparations of chicken heart mitochondrial malic dehydrogenase and six preparations of the supernatant enzyme using from 20 to 600 lb of chicken hearts per preparation. The enzymes have been prepared both by prior isolation of mitochondrial and supernatant fractions and from total tissue extracts, with later separation of the two enzymes. The different means of preparation did not lead to any detectable differences in the properties of the enzymes. Since we normally attempt to purify several enzymes other than malic dehydrogenases from a batch of tissue and since frozen tissues are more readily available than fresh material, our preferred procedure has been to use total tissue extracts and later separate the mitochondrial and supernatant malic dehydrogenases by column chromatography. The following is a typical purification procedure, using a 20-lb batch of chicken hearts, this being the most convenient amount to handle in the laboratory.

STEP 1. CRUDE EXTRACT. Frozen chicken hearts (20 lb), stripped of fat, were ground three times in a mechanical meat grinder. The minced tissue was then suspended in 10 l. of 0.005 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.5, for 1 hr at 4° with occasional stirring. After this time the suspension was homogenized, in batches, in a large Waring blendor (30 sec/batch) and allowed to stand in the cold with continuous stirring for an additional 2 hr. The homogenate was filtered through several layers of cheesecloth, placed in 5 l. of the above buffer, and stirred in the cold for 1 hr. After the extracted homogenate was again passed through cheesecloth the solid residue was discarded and the two filtrates were combined. The combined filtrates were clarified by centrifugation for 30 min at 1300*g* at 4° and the residues were discarded. Starch gel electrophoresis at pH 7.0 showed that both mitochondrial and supernatant malic dehydrogenases had been extracted.

STEP 2. FIRST AMMONIUM SULFATE PRECIPITATION. Solid ammonium sulfate was added to the combined filtrates to give 40% saturation.[2] In this and in other ammonium sulfate precipitations the pH was maintained at 7.5 by addition of ammonium hydroxide. The suspension was left at 4° for several hours and then filtered overnight on fluted filter papers. The precipitate was discarded. More solid ammonium sulfate was added to the filtrate to give 85% saturation. The suspension was left at 4° for 2 hr and then centrifuged at 1300*g* for 30 min. The precipitate was dissolved in 0.005 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.5. Most of the malic dehydrogenase activity was present in the precipitate. The supernatant was discarded.

STEP 3. SECOND AMMONIUM SULFATE PRECIPITATION. The dissolved precipitate from step 2 was dialyzed against three changes of 0.005 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.5, 20 l., about 6 hr for each change. After dialysis the enzyme solution was clarified by centrifuging at 20,000*g* for 15 min. To the clear supernatant, solid ammonium sulfate was added to give 50% saturation. After 2 hr at 4° the suspension was centrifuged at 20,000*g* for 15 min. The precipitate contained only a small fraction of the total malic dehydrogenase activity and was discarded. Further solid ammonium sulfate was added to give 80% saturation. The suspension was left at 4° for 2 hr and centrifuged as before. The precipitate was dissolved in 0.05 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.5, and dialyzed at 4° against three changes of the same buffer (6 l.), 6 hr/change. Starch gel electrophoresis at pH 7.0 showed the presence of both mitochondrial and supernatant malic dehydrogenases.

STEP 4. NEGATIVE ADSORPTION ON DEAE-CELLULOSE. By means of test-tube scale experiments it was found that neither supernatant nor mitochondrial malic dehydrogenase was adsorbed to DEAE-cellulose at pH 7.5 in 0.05 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, although a considerable amount of other proteins was retained by the resin under these conditions. DEAE-cellulose was prepared as described by Pesce *et al.* (1964), except that the equilibration steps were carried out in 0.5 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.5, and finally in 0.05 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.5. The dialyzed enzyme solution from step 3 was placed on a DEAE-cellulose column (4.5 × 50 cm) and eluted with the buffer used for the last change

[2] Per cent saturation was based on Table I in Green and Hughes (1955) even though the enzyme solutions were kept at 4°.

of dialysis. The malic dehydrogenases were not retained on the column. The fractions containing malic dehydrogenase activity were combined and dialyzed overnight against saturated ammonium sulfate–0.01 M β-mercaptoethanol, pH 7.5, to concentrate the enzyme. The precipitated enzymes were centrifuged at 20,000*g* for 15 min, and the precipitate was dissolved in 0.005 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 6.5, and dialyzed overnight at 4° with three changes (6 l.) of the same buffer.

STEP 5. CARBOXYMETHYLCELLULOSE CHROMATOGRAPHY. Carboxymethylcellulose was prepared as described by Pesce *et al.* (1964), except that the equilibration steps were carried out in 0.5 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 6.5, and finally in 0.005 M potassium phosophate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 6.5. The dialyzed enzyme solution from step 4 was placed on a 4.5 × 50 cm carboxymethylcellulose column.

The supernatant enzyme was not adsorbed to the resin and was eluted from the column with 0.005 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 6.5. The fractions containing malic dehydrogenase activity were combined and concentrated by dialysis against saturated ammonium sulfate as described above. This fraction (CMC-1) was shown by starch gel electrophoresis to contain only the supernatant form of malic dehydrogenase which was then further purified as described below.

The mitochondrial malic dehydrogenase was adsorbed to the carboxymethylcellulose and was eluted with a linear gradient established between 2 l. of 0.005 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 6.5, and 2 l. of 0.2 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 6.5. The fractions containing malic dehydrogenase activity were combined and concentrated by dialysis against saturated ammonium sulfate, pH 7.5, as described above. Starch gel electrophoresis showed this fraction (CMC-2) to contain only the mitochondrial malic dehydrogenase. This enzyme was further purified as described below.

STEP 6. PARTIAL CRYSTALLIZATION OF CHICKEN HEART SUPERNATANT MALIC DEHYDROGENASE. The supernatant enzyme (fraction CMC-1 from step 5), after ammonium sulfate concentration, was dialyzed overnight, at 4°, against three changes (6 l.) of 0.005 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.8. The enzyme solution was then placed on a 4.5 × 40 cm column of DEAE-cellulose previously equilibrated in the same buffer and eluted with a linear gradient established between 2 l. of 0.005 M potassim phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.8, and 2 l. of 0.2 M potassium-phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol, pH 7.8. The enzyme was eluted at a salt concentration of approximately 0.025 M[3] and fractions containing malic dehydrogenase activity were combined and concentrated by dialysis against saturated ammonium sulfate as described above. At this stage fractional addition of ammonium sulfate to a dialyzed sample of the enzyme yielded a partly crystalline suspension. However, when examined in the ultracentrifuge, such a sample showed a minor contaminant which sedimented faster than the major peak of malic dehydrogenase. This contaminant wasr emoved by gel filtration as described below.

STEP 7. GEL FILTRATION OF CHICKEN SUPERNATANT MALIC DEHYDROGENASE. The concentrated enzyme solution in ammonium sulfate obtained in step 6 was dialyzed for 6 hr at 4° against three changes (3 l.) of 0.05 M Tris-HCl buffer, pH 7, containing 0.1 M KCl and 0.001 M β-mercaptoethanol. The enzyme solution (approximately 15 ml) was then placed on a 2.5 × 80 cm column of Sephadex G-100, previously equilibrated with the same buffer. Two well-separated protein fractions were eluted, only the major peak having malic dehydrogenase activity. The fractions having the highest malic dehydrogenase activity were combined and concentrated by dialysis against saturated ammonium sulfate as described above. The leading and trailing edges of the malic dehydrogenase peak were discarded.

STEP 8. CRYSTALLIZATION OF CHICKEN SUPERNATANT MALIC DEHYDROGENASE. The concentrated enzyme solution from step 7 was dialyzed for 6 hr against three changes (2 l.) of 0.1 M PO_4–0.001 M EDTA–0.005 M β-mercaptoethanol, pH 7.5. Solid ammonium sulfate was added to give 50% saturation and the very small amount of amorphous, inactive protein precipitated was removed by centrifugation. Additional solid ammonium sulfate was added slowly to the solution over a period of 5 hr until slight turbidity was observed at an ammonium sulfate concentration of approximately 65%. The enzyme solution was stored at 4° and the enzyme began to crystallize after about 8 hr. Crystallization was allowed to continue at this temperature for 3 days, after which time the crystals were harvested and recrystallized four times in the same manner. The yield was 200 mg of crystalline enzyme representing some 15% of the total malic dehydrogenase activity present in the crude tissue extract.

STEP 9. FURTHER PURIFICATION OF CHICKEN MITOCHONDRIAL MALIC DEHYDROGENASE. The mitochondrial malic dehydrogenase fraction obtained from carboxymethylcellulose chromatography (fraction CMC-2 from step 5) was dialyzed against 0.1 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol (three changes of 3 l. each). Solid ammonium sulfate was added to 45% saturation and the solution was clarified by centrifugation. Additional ammonium sulfate was slowly added over a period of 4 hr and crystallization began to occur at approximately 55% saturation. The enzyme was allowed to crystallize over a period of 3 days and the crystals were harvested

[3] We have experienced some difficulty in absorbing the chicken supernatant malic dehydrogenase on DEAE-cellulose under these conditions unless the resin is thoroughly washed and fully equilibrated prior to use.

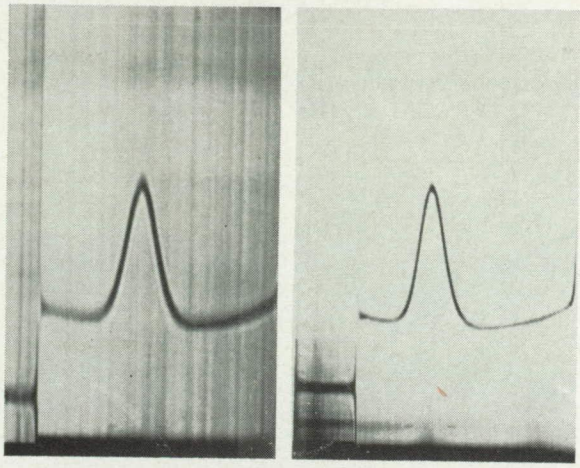

FIGURE 1: Sedimentation velocity patterns of crystalline mitochondrial (left) and supernatant (right) malic dehydrogenases. Protein concentration in both cases was approximately 12 mg/ml in 0.1 M phosphate buffer, pH 7.0. The rotor speed was 59,780 rpm. The temperatures were 23.5 and 21.25° and the bar angles 70 and 60° for the mitochondrial and supernatant enzymes, respectively.

by centrifugation. Ultracentrifugal analysis revealed the presence of a very minor contaminant and the specific activity of the enzyme was somewhat below that reported by Thorne (1962) for pig mitochondrial malic dehydrogenase. Repeated crystallizations failed to significantly increase the specific activity, and fluorescence spectroscopy revealed the presence of tryptophan, an amino acid absent from the pig mitochondrial enzyme. The enzyme preparation was rechromatographed on carboxymethylcellulose as described in step 5, this time using 4 l. of buffer in each chamber of the linear gradient mixing device. The enzyme was then subjected to gel filtration on Sephadex G-100 as described in step 7 for the supernatant enzyme. The most active fractions were combined and concentrated by dialysis against saturated ammonium sulfate.

STEP 10. CRYSTALLIZATION OF CHICKEN MITOCHONDRIAL MALIC DEHYDROGENASE. The concentrated enzyme solution obtained after gel filtration was dialyzed against three changes (3 l.) of 0.1 M potassium phosphate–0.001 M EDTA–0.001 M β-mercaptoethanol. Solid ammonium sulfate was added slowly over a period of 5 hr and crystallization began at approximately 55% saturation. After remaining at 4° for 2 days the crystals were harvested by centrifugation and recrystallized four times in the same manner. The yield was 160 mg of crystalline enzyme, which represented approximately 11% of the total malic dehydrogenase activity of the crude tissue extract. From a 600-lb batch of chicken hearts we were able to obtain approximately 5 g of both supernatant and mitochondrial malic dehydrogenases, using essentially the procedure described above.

Concurrent with the isolation of these malic dehydrogenases Dr. Linda Bertland, of this laboratory, was purifying chicken supernatant and mitochondrial aspartate aminotransferases from the same starting material. It became apparent in the later stages of purification that these aminotransferases were the major contaminants in the malic dehydrogenase preparations. Both types of enzyme were eluted together from carboxymethylcellulose. Separation of malic dehydrogenases and aspartate aminotransferases was accomplished in the steps involving ammonium sulfate precipitation and most effectively by gel filtration. The final crystalline supernatant and mitochondrial malic dehydrogenases contained less than 0.05% aminotransferase activity as impurity.

Results

Ultracentrifugal Studies and Physiochemical Characteristics. All crystalline preparations of the chicken mitochondrial and supernatant malic dehydrogenases were examined in the ultracentrifuge using schlieren optics and all were shown to sediment as a single symmetrical peak. Typical examples are shown in Figure 1. Sedimentation coefficients corrected to 20° and water were calculated from twelve ultracentrifuge runs for the mitochondrial enzyme and ten runs for the supernatant enzyme. All runs were carried out in 0.1 M potassium phosphate–0.001 M EDTA, pH 7.5, at a protein concentration of approximately 10 mg/ml. The mean $s_{20,w}$ value for the mitochondrial enzyme was 4.1 ± 0.1 and for the supernatant enzyme 4.1 × 0.15. The concentration dependence of the $s_{20,w}$ value for samples of the mitochondrial and supernatant enzymes is presented in Figure 2. A slight increase in $s_{20,w}$ value with dilution was noted

TABLE 1: Physiochemical Characteristics of Chicken Heart Mitochondrial and Supernatant Malic Dehydrogenases.[a]

	Mitochondrial	Supernatant
Sedimentation coefficient ($s^0_{20,w} \times 10^{-13}$ cm sec^{-1})	4.30	4.27
Diffusion coefficient ($D_{20,w} \times 10^7$ cm^2 sec^{-1})	5.44	
Frictional ratio f/f_0 from $s_{20,w}$ and $D_{20,w}$	1.4	
Molecular weight from S/D	72,800	
Molecular weight from S and D	67,000	
Molecular weight from sedimentation equilibrium		66,500
Molecular weight from gel filtration	67,000	67,000

[a] See Materials and Methods for details.

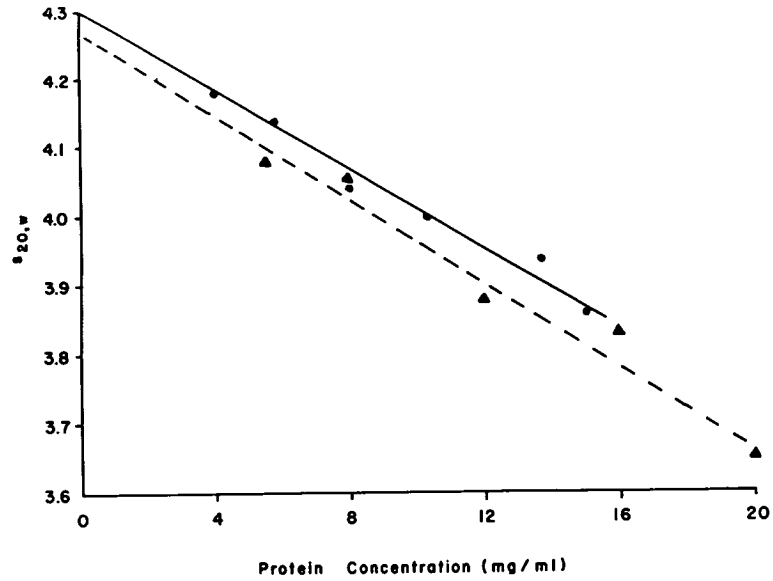

FIGURE 2: Plots showing the concentration dependence of the sedimentation coefficients of chicken mitochondrial (●) and supernatant (▲) malic dehydrogenases.

in both cases. Extrapolated $s_{20,w}$ values were 4.3 S for the mitochondrial enzyme and 4.3 S for the supernatant enzyme. These values are in good agreement with those reported for the pig, horse, and ox heart enzymes by Thorne (1962). The results presented in Table I indicate that both supernatant and mitochondrial malic dehydrogenases are of similar, if not identical, molecular weight. This is supported by the fact that both supernatant and mitochondrial malic dehydrogenases have an identical elution volume, corresponding to a molecular weight of 67,000 when run on Sephadex G-100 columns calibrated for molecular weight determination (Andrews, 1964). This is in agreement with a similar finding for the pig supernatant and mitochondrial malic dehydrogenases (Thorne and Cooper, 1963). The diffusion constant obtained for the chicken mitochondrial enzyme (5.44×10^{-7} cm^2 sec^{-1}) is in close agreement with that found by Thorne and Kaplan (1963) for the pig mitochondrial enzyme. The frictional ratio, f/f_0, of 1.4 obtained for the chicken mitochondrial enzyme is identical with that calculated by Thorne and Kaplan (1963) for pig mitochondrial malic dehydrogenase. Further ultracentrifugal studies, concerned with reversible dissociation in acid of the chicken supernatant and mitochondrial malic dehydrogenases, have been reported elsewhere (Chilson *et al.*, 1965, 1966).

Extinction Coefficients. The extinction coefficients at 280 mμ for these enzymes were determined by measuring the absorbance at 280 mμ in 0.1 M potassium phosphate, pH 7.5, of a carefully dialyzed and clarified solution of the enzyme and then assaying the nitrogen content of aliquots of the same solution by the Kjeldahl method. By using a value of 16% nitrogen for these proteins, the $E_{1cm}^{1\ mg/ml}$ at 80 mμ was then determined and from this a molecular extinction coefficient (E_m) was calculated. Nominal molecular weights of 67,000 were used for both enzymes. The extinction coefficient of chicken mitochondrial malic dehydrogenase was also determined by comparing solutions of the enzyme, of known absorbance at 280 mμ, with known concentrations of bovine serum albumin, using the Biuret reaction. The published extinction coefficient of bovine serum albumin, $E_{1cm}^{1\%}$ of 6.67 (Foster and Sherman, 1956), was then used to calculate the extinction coefficient of the malic dehydrogenase. The results are presented in Table II.

TABLE II: Extinction Coefficients at 280 mμ of Chicken Supernatant and Mitochondrial Malic Dehydrogenases.

	Mitochondrial	Supernatant
$E_{1cm}^{1\%}$ (Kjeldahl)	2.9	13.1
$E_{1cm}^{1\%}$ (Biuret)	3.1	
E_m (m = 67,000) (M^{-1} $cm^{-1} \times 10^{-4}$)	1.95	8.7

The extinction coefficient obtained for chicken mitochondrial malic dehydrogenase is in good agreement with that obtained by Thorne and Kaplan (1963) for the pig mitochondrial enzyme and reflects the lack of tryptophan in both enzymes. The extinction coefficients of other crystalline supernatant malic dehydrogenases have not been reported.

Amino Acid Compositions. The results presented in Table III were obtained with at least four analyses

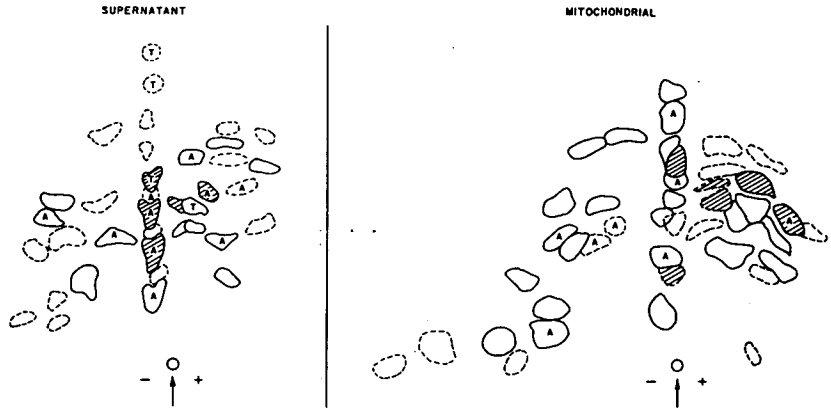

FIGURE 3: Peptide maps of tryptic digests of chicken mitochondrial and supernatant malic dehydrogenases. Arginine-containing peptides are marked A and tryptophan-containing peptides, T. The sulfhydryl-containing peptides labeled with [^{14}C]iodoacetate are cross hatched.

TABLE III: Amino Acid Compositions of Mitochondrial and Supernatant Malic Dehydrogenases.

	Mitochondrial		Supernatant	
	Chicken	Pig[a]	Chicken	Pig[b]
Lys	49 ± 4.2[c]	57	57 ± 3.4[c]	57
His	11 ± 1.6	13	11 ± 1.4	10
Arg	20 ± 2.2	15	20 ± 2.4	19
Asp	52 ± 3.1	49	68 ± 2.7	70
Thr	44 ± 3.8	46	30 ± 3.2	29
Ser	42 ± 3.7	39	39 ± 3.4	42
Glu	60 ± 3.4	48	60 ± 1.2	59
Pro	41 ± 1.4	52	29 ± 2.1	27
Gly	61 ± 2.1	62	59 ± 2.2	48
Ala	66 ± 1.1	74	60 ± 1.6	61
½-Cys	14[d]	—	8[d]	—
Val	48 ± 1.7	51	53 ± 3.1	51
Met	13 ± 1.2	11	14 ± 1.1	15
Ile	40 ± 0.8	40	42 ± 2.3	40
Leu	57 ± 1.6	58	59 ± 2.5	60
Tyr	9 ± 2.1	11	16 ± 1.1	15
Phe	30 ± 0.9	22	24 ± 0.7	24
Trp	0[d]	0[d]	12[d]	16[d]

[a] Data from Thorne (1962). [b] Data from Thorne and Cooper (1963). [c] Standard deviation. [d] See Materials and Methods for details of method of analysis. All values are reported as residues per mole (67,000 mol wt).

of three different preparations of the mitochondrial enzyme and two preparations of the supernatant enzyme. There was a slight increase in the yield of valine and isoleucine between the 24- and 48-hr hydrolysates. The higher values were chosen to represent complete liberation of the residues. Other residues were extrapolated to zero time of hydrolysis. If no extensive destruction of serine and threonine occurred between the 24- and 48-hr hydrolysates, the amino acids were corrected to zero time of hydrolysis by dividing the 24-hr values by 0.90 for serine and 0.95 for threonine, these correction factors having been established from previous work in this laboratory (Pesce *et al.*, 1964). The data of Thorne (1962) and Thorne and Cooper (1963) for pig mitochondrial and supernatant malic dehydrogenases are included for comparison. The mitochondrial enzymes of pig and chicken are strikingly similar in amino acid composition, as are the pig and chicken supernatant enzymes. There are, however, marked differences in composition between the mitochondrial and supernatant enzymes, the most striking of these being the complete absence of tryptophan in the mitochondrial enzymes. Crystalline mitochondrial malic dehydrogenase, prepared in this laboratory from yellow fin tuna, has also been found to be devoid of tryptophan.

Peptide Mapping. Tryptic digestion and subsequent peptide mapping of chicken mitochondrial and supernatant malic dehydrogenase was carried out by methods previously used in this laboratory to study lactic dehydrogenases (Fondy *et al.*, 1964). Peptide maps of the chicken supernatant and mitochondrial malic dehydrogenases are shown in Figure 3. When the peptide maps of the enzymes were treated with Erlich's stain for tryptophan (Smith, 1960), none of the spots on the map of the mitochondrial enzyme gave a positive reaction, confirming the absence of tryptophan in this enzyme.

Peptide maps of tryptic digests of the mitochondrial enzyme gave between 27 and 32 ninhydrin-positive spots, while maps of the supernatant enzyme gave between 33 and 39 spots. In both cases the number of spots observed is slightly less than one-half the number expected on the basis of the total lysine and arginine content of the enzymes (see Table III). These data suggest that both enzymes are dimeric in the native state, a finding consistent with our earlier studies on the acid dissociation of these enzymes

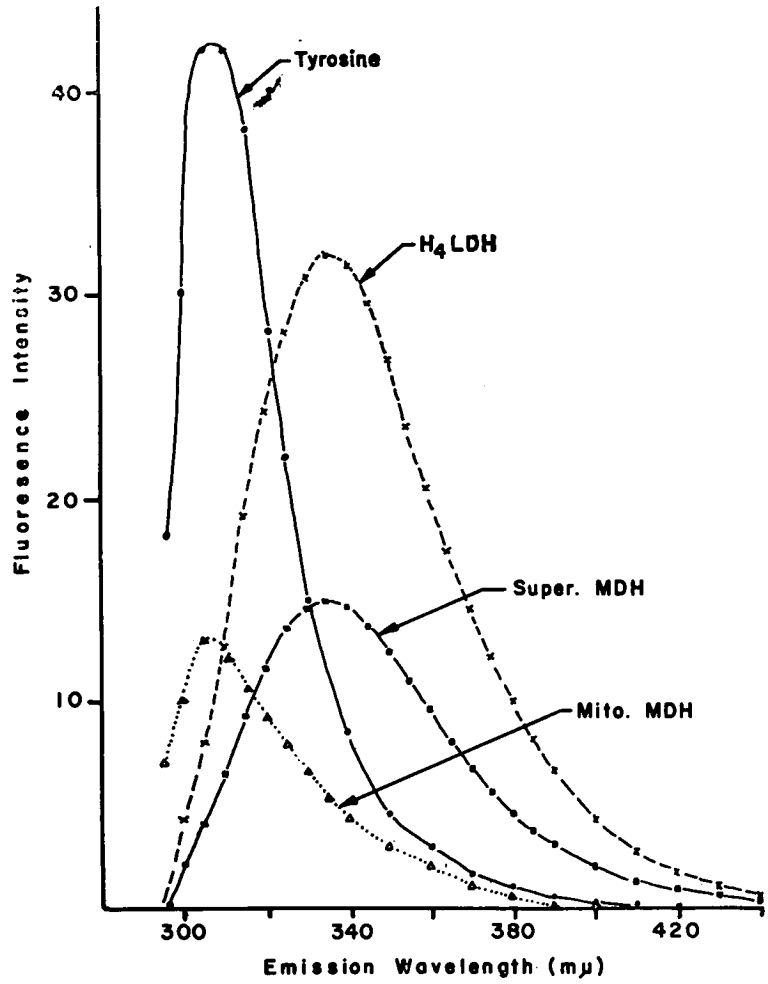

FIGURE 4: Fluorescence emission spectra of solutions of tyrosine, mitochondrial and supernatant chicken malic dehydrogenase, and lactic dehydrogenase in 0.1 M phosphate buffer, pH 7.5. All measurements were made in a Zeiss spectrofluorometer Model ZFM 4c at 25°, the excitation wavelength being 280 mμ. (△), chicken heart mitochondrial malic dehydrogenase (MDH), $A_{280\ m\mu} = 0.1$; (■), chicken heart supernatant malic dehydrogenase, $A_{280m\mu} = 0.008$; (×), chicken heart lactic dehydrogenase (H_4LDH), $A_{280m\mu} = 0.009$; (●), tyrosine, $A_{280m\mu} = 0.02$. Fluorescence intensity in arbitrary units.

(Chilson *et al.*, 1965, 1966). Although little can be said about the neutral peptides, because of overlaps in the peptide maps, it is apparent that there are few common basic or acidic peptides in the chicken mitochondrial and supernatant malic dehydrogenases. This is similar to the findings reported by Dévényi *et al.* (1966) for pig malic dehydrogenases.

Preliminary experiments showed that it was not possible to selectively label the sulfhydryl groups of the malic dehydrogenases by treatment with iodoacetate, as had been possible with lactic dehydrogenases (Fondy *et al.*, 1965). Consequently, it was decided that the most practical way of labeling sulfhydryl groups was by treatment of the native enzymes with iodoacetate in urea. The chicken supernatant and mitochondrial malic dehydrogenases were incubated under nitrogen for several hours with radioactive [^{14}C]iodoacetate in 8.0 M urea using a ratio of 5 moles of iodoacetate/sulfhydryl group. Unbound iodoacetate was removed by dialysis and the labeled mixture was digested with trypsin. Peptide maps were made using the same method used for unlabeled protein. The radioactive spots were located by autoradiography of the peptide maps. The peptides containing labeled sulfhydryl groups are indicated in Figure 3. Further studies on these sulfhydryl-containing peptides, and on such peptides from a number of other malic dehydrogenases, will be presented elsewhere.

Fluorescence Studies. The fluorescence emission spectra of the chicken mitochondrial and supernatant malic dehydrogenases are shown in Figure 4. The spectrum of the chicken mitochondrial malic dehydrogenase is very similar to those obtained by Thorne and Kaplan (1963) for the pig and horse mitochondrial enzymes, with a fluorescence maximum at 307 mμ. Such a spectrum corresponds to that of free tyrosine and is typical of proteins devoid of tryptophan (Teale, 1960). The chicken supernatant enzyme, which, unlike the mitochondrial enzyme, contains a number of tryptophan residues, has a fluorescence spectrum

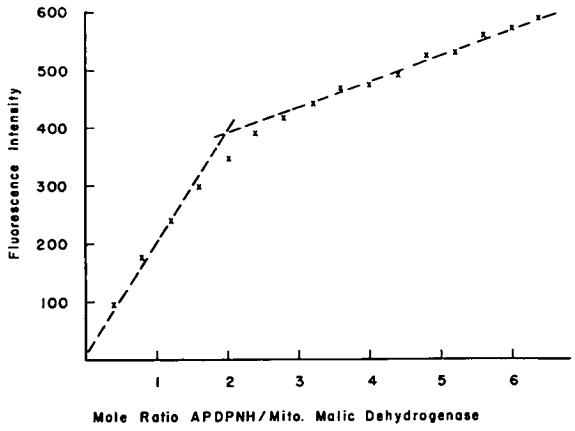

FIGURE 5: Fluorescence titration of chicken mitochondrial malic dehydrogenase with APDPNH in 0.1 M sodium phosphate (pH 7.5). Aliquots (10 μl) of coenzyme were added to a 2-ml solution of enzyme (enzyme concentration = 3.14×10^{-6} M). Excitation was at 363 mμ and emission was measured at 450 mμ. Fluorescence intensity in arbitrary units. A nomina molecular weight of 70,000 was assumed for the enzyme.

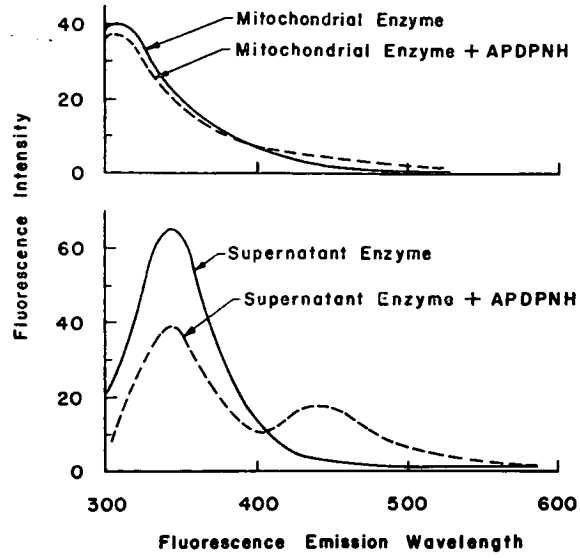

FIGURE 6: A comparison of the protein fluorescence changes accompanying binding of APDPNH to chicken mitochondrial (top) and supernatant (bottom) malic dehydrogenases. APDPNH = 7×10^{-6} M; mitochondrial malic dehydrogenase = 1.7×10^{-6} M; supernatant malic dehydrogenase = 0.9×10^{-6} M. Excitation was at 288 mμ; fluorescence intensity in arbitrary units.

with a maximum at 335 mμ, when excited at 280 mμ, like that of chicken heart lactic dehydrogenase, another tryptophan-containing protein.

Fluorescence changes accompanying the binding of reduced coenzyme to the supernatant and mitochondrial malic dehydrogenases reflect the difference in tryptophan content of the two enzymes. As illustrated in Figure 5, binding of the reduced acetylpyridine analog of DPN^+ to the supernatant enzyme causes a marked decrease in the protein fluorescence at 335 mμ and the appearance of a new fluorescence peak at 440 mμ when the protein is excited at 280 mμ. By contrast, when the reduced coenzyme analog binds to the mitochondrial enzyme there is less quenching of the protein fluorescence peak at 307 mμ and a negligible increase in 440-mμ fluorescence when excited at 280 mμ. In the case of the tryptophan-containing supernatant enzyme, the overlap of the tryptophan emission spectrum with the absorption spectrum of the reduced coenzyme analog (λ_{max} 365 mμ) is sufficient to allow considerable transfer of the excitation energy from protein to coenzyme. This is not possible in the tryptophan-free mitochondrial enzyme, and the minimal overlap of the tyrosine emission spectrum with the absorption spectrum of the reduced coenzyme analog allows of less transfer of excitation energy.

The stoichiometry of the binding of the reduced acetylpyridine analog of DPN^+ to the chicken supernatant and mitochondrial malic dehydrogenases was determined essentially by the method of Velick (1958). Reduced coenzyme (1 mole) was found to be bound to 35,000 and 34,000 g of the mitochondrial and supernatant enzymes, respectively. A typical experiment showing the binding of the coenzyme analog to the mitochondrial enzyme is illustrated in Figure 6.

The formation of a ternary complex between chicken mitochondrial malic dehydrogenase, the reduced acetylpyridine analog of DPN^+, and L-malate was demonstrated by measuring the increase in fluorescence of the binary complex (excitation 365 mμ; emission at 460 mμ) on addition of small amounts of L-malate, as described by Thorne and Kaplan (1963). The enzyme concentration was 0.23 mg/ml, the coenzyme concentration was 2.7×10^{-6} M, and the final L-malate concentration was 0.14 M. The dissociation constant of L-malate from the ternary complex was found to be 6.1 mM, which is comparable to the figure of 6.7 mM reported by Thorne and Kaplan (1963) for pig mitochondrial malic dehydrogenase.

The polarization of fluorescence of a solution containing chicken mitochondrial malic dehydrogenase and DPNH was measured by the method of McKay and Kaplan (1964) using an instrument constructed in this laboratory and described by the above authors. The enzyme concentration was 0.25 mg/ml and the DPNH concentration 2.7×10^{-6} M. The polarization of fluorescence of this solution was 0.454, while that of coenzyme alone, at the same concentration, was 0.0866. Most of the polarization is therefore due to the presence of a mitochondrial malic dehydrogenase–DPNH binary complex. McKay and Kaplan (1964) reported a value of 0.27 for the polarization of fluorescence of a chicken heart lactic dehydrogenase–DPNH complex. The difference in polarization between the two complexes may indicate differences in the environment or in the mode of association of DPNH in these two enzymes.

Starch Gel Electrophoresis. Although both the supernatant and mitochondrial chicken malic dehydrogenases appear to be homogeneous by ultracentrifugal analysis, and by a number of other criteria, when these enzymes are subjected to electrophoresis on starch gels both enzymes show several enzymatically active bands, as illustrated in Figure 7. Such multiple forms have previously been noted with a number of mitochondrial malic dehydrogenases (Thorne *et al.*, 1963; Henderson, 1964) and with pig supernatant malic dehydrogenase (Kulick and Barnes, 1965).[4]

We have recently reported elsewhere (Kitto *et al.*, 1966a,b) that the multiple electrophoretic forms of chicken mitochondrial malic dehydrogenase result solely from conformational differences rather than from differences in primary structure as is the case with the multiple forms of lactic dehydrogenase (Cahn *et al.*, 1962; Fondy *et al.*, 1964) and with creatine kinase (Dawson *et al.*, 1965). The nature of the multiple forms of chicken supernatant malic dehydrogenase has not been further investigated.

Immunological Properties. The immunization of rabbits with chicken mitochondrial and supernatant malic dehydrogenases yielded potent antisera to both enzymes, which were characterized as described in Materials and Methods. When tested by double diffusion in agar (Ouchterlony, 1948), the antisera to chicken mitochondrial malic dehydrogenase gave a single, sharp precipitin band with this enzyme but no detectable cross-reaction with the chicken supernatant enzyme. Antisera to chicken supernatant malic dehydrogenase showed exactly the opposite behavior, reacting well with the supernatant enzyme but not at all with the mitochondrial enzyme. Similar results were obtained when the antisera were tested by immunoelectrophoresis. The data presented in Table IV indicate that antibodies to the mitochondrial malic dehydrogenase are capable of effectively inhibiting this enzyme, while they have no effect on the activity of the supernatant enzyme. The reverse is true of antibodies to the supernatant enzyme.

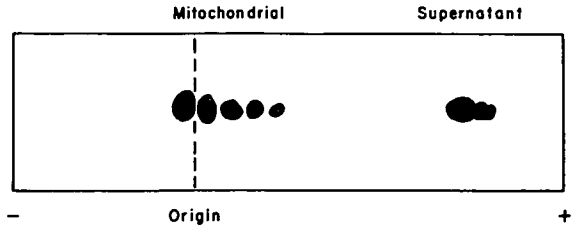

FIGURE 7: A tracing of a starch gel electrophoresis of chicken mitochondrial and supernatant malic dehydrogenases. The electrophoresis was carried out in phosphate–citrate buffer, pH 7.0, at 10 v/cm for 16 hr at 4°.

The quantitative, and highly sensitive, technique of microcomplement fixation (Wasserman and Levine, 1961) offered a means of examining in detail the immunological reactivity of these antisera. Using this technique, it was found that although the antisera to

TABLE IV: Inhibition by Antibody of Chicken Mitochondrial and Supernatant Malic Dehydrogenases.[a]

Enzyme	Antimitochondrial Serum (% inhibn)	Antisupernatant Serum (% inhibn)
Mitochondrial	59	0
Supernatant	0	40

[a] The enzymes were incubated at 0° for 15 min with 1:150 dilutions of anitsera. See text for details.

TABLE V: Complement Fixation with Antiserum to Chicken Mitochondrial Malic Dehydrogenase.

Species	Rel Antiserum Concn Needed for 50% C′ Fixation[a]
Chicken (*Gallus gallus*)	1.0
Mallard duck (*Anas platyrhynchos*)	1.2
Ostrich (*Struthio camelus*)	1.3
Hoopoe (*Upupa epops*)	1.9
Blue jay (*Cyanocitta cristata*)	2.8
Bullfrog (*Rana catesbiana*)	4
Caiman (*Caiman crocodilus*)	8
Painted turtle (*Chrysemys picta*)	8
Iguana (*Iguana iguana*)	11
Water snake (*Natrix* sp.)	20
Boa constrictor (*Constrictor constrictor*)	20
Sturgeon (*Acipenser transmontanus*)	20
Pig (*Sus scrofa*)	40
Tuna (*Neothunnus macropterus*)	80
Lobster (*Homarus americanus*)	>120

[a] The antibody to chicken mitochondrial malic dehydrogenase cross-reacts only with mitochondrial malic dehydrogenases and not with the supernatant enzymes. Crude extracts were used as a source of the mitochondrial enzyme. The presence of supernatant enzyme in a tissue extract does not affect the complement fixation procedure.

[4] The multiple forms of chicken supernatant malic dehydrogenase are not well resolved using phosphate–citrate buffer. A Tris–maleate system gives better resolution (R. J. Kulick, personal communication).

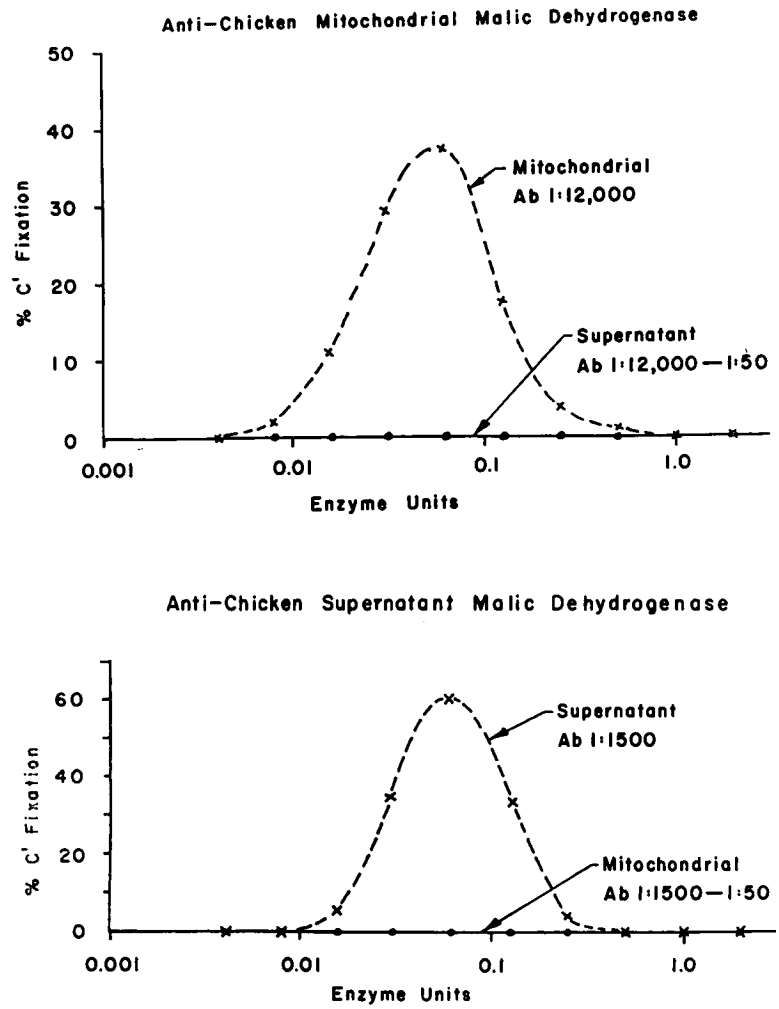

FIGURE 8: Complement fixation experiments showing (top) the lack of cross-reaction between an antichicken mitochondrial malic dehydrogenase serum and chicken supernatant malic dehydrogenase. The antibody dilution for the reaction with chicken mitochondrial malic dehydrogenase was 1:12,000. No cross-reaction was observed with the chicken supernatant malic dehydrogenase with a range of antibody dilution from 1:12,000 to 1:50; (bottom) the lack of cross-reaction between an antichicken supernatant malic dehydrogenase serum and chicken mitochondrial malic dehydrogenase. The antibody dilution for the reaction with chicken supernatant malic dehydrogenase was 1:1500. No cross-reaction was observed with the chicken mitochondrial malic dehydrogenase with a range of antibody dilution from 1:150 to 1:50. See Wasserman and Levine (1961) for details of complement fixation.

the mitochondrial malic dehydrogenase could react with the mitochondrial enzyme at dilutions of antibody as high as 1:12,000 (Ra 383B3-5), this same antibody showed no cross-reaction with the supernatant enzyme, even with dilutions of antibody as low as 1:50, over a wide range of enzyme concentration. The reverse was true of the antisera to chicken supernatant malic dehydrogenase (Figure 8). Additions of high concentrations (100-fold excess) of the supernatant enzyme, to a system containing mitochondrial enzyme and antimitochondrial antibody, failed to produce any inhibition of the homologous complement fixation reaction. Neither was inhibition observed when mitochondrial enzyme was added to a supernatant enzyme–antisupernatant antibody system. Similar patterns of immunological reactivity were found with rabbit antisera directed against crystalline tuna and pig mitochondrial malic dehydrogenases and against crystalline ostrich supernatant malic dehydrogenase.

Antisera to the chicken supernatant and mitochondrial malic dehydrogenases do show cross-reactions with their respective forms of enzyme from species other than chicken. Typical examples of such cross-reactions, using the microcomplement fixation technique, are shown in Table V for an antichicken mitochondrial malic dehydrogenase antiserum. The extent of the cross-reaction, in this system, as well as a number of others (see Wilson and Kaplan, 1964), appears to be directly related to the taxonomic distance be-

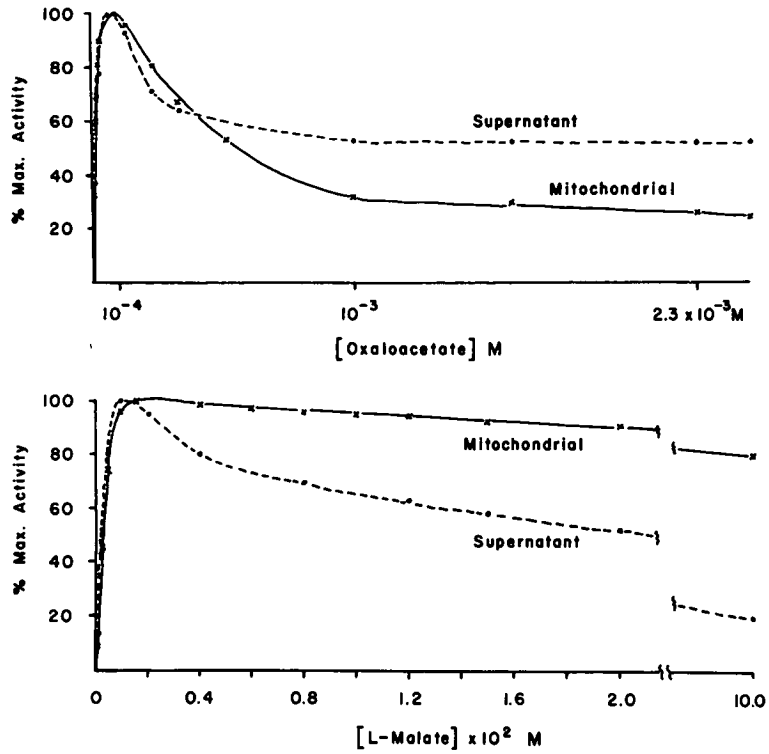

FIGURE 9: The relationship between enzymatic activity and the concentrations of malate and oxaloacetate with chicken mitochondrial and supernatant malic dehydrogenases.

TABLE VI: Catalytic Characteristics of Chicken Mitochondrial and Supernatant Malic Dehydrogenases.

	Mitochondrial Enzyme	Supernatant Enzyme
K_m malate	9×10^{-4} M	8×10^{-4} M
K_m oxaloacetate	3.8×10^{-5} M	5×10^{-5} M
Sp act., units/ml A_{280} (malate, DPN^+)[a]	760	85
Sp act., units/mg (malate, DPN^+)[b]	260	65
pH optima (malate oxidation)	10.0	10.0
pH optima (oxaloacetate reduction)	7.8	7.6

[a] Assay procedure and nomenclature of Thorne (1962). [b] From the experimentally determined extinction coefficients.

tween the species involved. An exception to such a rule is when a species is closely related to the animal used for immunization. Enzymes from such animals typically give much weaker cross-reactions than would be expected from their taxonomic relationship to the animal used in obtaining the immunizing antigen. This is typified, in the present instance, by the weak cross-reaction of the pig enzyme since a rabbit antibody was used (Table V). Results of a more detailed toxonomic investigation of this nature will be presented elsewhere.

Catalytic Properties. A number of the catalytic properties of the chicken supernatant and mitochondrial malic dehydrogenases are summarized in Table VI. The specific activity of the mitochondrial enzyme is similar to those reported by Thorne and Kaplan (1963) for the mitochondrial enzymes of pig and horse, while the specific activity of the chicken supernatant enzyme is comparable to that obtained by Thorne and Cooper (1963) for the pig supernatant enzyme.

The Michaelis constants of the chicken enzymes for malate and oxaloacetate are similar to those reported for other malic dehydrogenases (Thorne, 1962; Englard and Breiger, 1962; Grimm and Doherty, 1961; Siegel and Englard, 1961). Neither enzyme showed any detectable enzymatic activity with D-malate. The supernatant and mitochondrial enzymes could readily be distinguished on the basis of their reactivity with a number of coenzyme analogs as shown in Table VII. With L-malate as substrate neither enzyme showed measurable reduction of TPN^+. With oxaloacetate as substrate the rate of oxidation of TPNH was, for both enzymes, less than 1% of that observed with DPNH.

As shown in Figure 9, the supernatant enzyme was markedly inhibited by high concentrations of L-

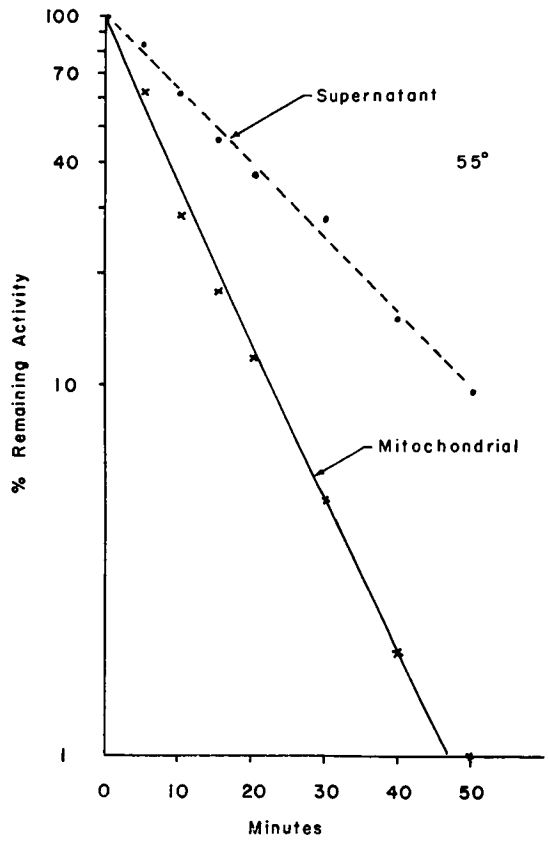

FIGURE 10: Rates of thermal inactivation of chicken mitochondrial and supernatant malic dehydrogenase at 55°. See Materials and Methods for details.

TABLE VII: Analog Ratios of Chicken Mitochondrial and Supernatant Malic Dehydrogenases.

	Mitochondrial	Supernatant
DPN^+_H:$APDPN_L$[a]	0.57	1.5
$APDPN_H$:$TNDPN_L$	17	4.3
DPN^+_H:$TNDPN_L$	3.8	1.1
$DeDPN_H$:DPN^+_L	0.21	0.36

[a] The analog ratios represent the relative rates of reduction of coenzyme or coenzyme analogs at malate concentrations of 6×10^{-3} (L) or 1×10^{-1} M (H).

malate. These substrate concentrations had relatively little effect on the activity of the mitochondrial enzyme. By contrast, the mitochondrial enzyme was more strongly inhibited by high concentrations of oxaloacetate than was the supernatant enzyme. In this respect, the chicken enzymes have properties similar to those reported for the enzymes from a number of other sources (Davies and Kun, 1957; Delbrück *et al.*, 1959a,b; Englard and Breiger, 1962; Siegel and Englard, 1961).

With the crystalline chicken enzymes, we have confirmed the earlier findings of Shonk and Boxer (1964), who showed, using cellular fractionation procedures, that rat supernatant malic dehydrogenase was readily inactivated by high concentrations of ethanol at room temperature, while the mitochondrial enzyme was relatively unaffected by such treatment.

The data presented in Figure 10 show that chicken mitochondrial malic dehydrogenase is more thermolabile than is the supernatant enzyme. The difference in thermostability of the two malic dehydrogenases is, however, much less marked than the differences in stability of the heart and muscle forms of chicken lactic dehydrogenase (Fondy *et al.*, 1964).

Discussion

Although a number of reports on the catalytic properties of malic dehydrogenases from animal tissues have been in good agreement, the sedimentation coefficients reported for these enzymes have ranged from 2.1 to 5.1, while estimates of the molecular weights have varied from 20,000 to 70,000. Recent reports of the molecular weights of pig and beef mitochondrial malic dehydrogenases have been 62,000, 65,000, and 70,000 (Siegel and Englard, 1961; Grimm and Doherty, 1961; Thorne and Kaplan, 1963), and the value of 67,000 presently reported as the molecular weight of the chicken mitochondrial enzyme falls within this range. Englard and Breiger (1962) reported a molecular weight of 52,000 for the beef supernatant malic dehydrogenase. However, Thorne and Cooper (1963) reported that both the supernatant and mitochondrial enzymes of pig behaved identically on gel filtration, indicating a close similarity in size. The chicken supernatant and mitochondrial malic dehydrogenases also behaved identically on gel filtration, and ultracentrifugal analysis indicates that they are of very similar, if not identical, molecular weight.

Both the fact that the chicken supernatant and mitochondrial malic dehydrogenases bind 2 moles of coenzyme/mole of enzyme, and the data from peptide mapping, suggest that both enzymes are composed of two subunits. This is supported by our earlier studies on the acid dissociation of these enzymes and by the finding that only one hybrid enzyme could be formed between the supernatant and mitochondrial enzymes of a single species or between mitochondrial enzymes of different species (Chilson *et al.*, 1965, 1966).

The amino acid compositions and peptide maps of the chicken mitochondrial and supernatant malic dehydrogenases show marked dissimilarities. Since sulfhydryl groups have been implicated in the mechanism of malic dehydrogenases (Pfleiderer *et al.* 1962), as well as a number of other dehydrogenases, it is of particular interest that the chicken supernatant enzyme has only about half the number of cysteine residues as does the mitochondrial enzyme. Like the pig,

horse, and tuna heart mitochondrial malic dehydrogenases, the chicken mitochondrial enzyme is devoid of tryptophan. Hence, although tryptophan has been suggested to play an important role in the enzymatic mechanism of other dehydrogenases (see, for example, Schellenberg, 1965) this is obviously not the case for these mitochondrial malic dehydrogenases.

The evidence presented here, using a variety of procedures, indicates that the chicken mitochondrial and supernatant malic dehydrogenases are immunologically distinct. Grimm and Doherty (1961) examined the immunological properties of beef supernatant and mitochondrial malic dehydrogenases. As in the present case, they found that an antibody directed against the supernatant enzyme would not inhibit or cross-react with the mitochondrial enzyme. They did, however, report a weak cross-reaction between an antibody directed against the mitochondrial enzyme and the supernatant enzyme. It is suggested that this discrepancy may have been due to a slight contamination of the beef mitochondrial enzyme, used for immunization, with supernatant enzyme. That their immunizing antigen was, in this case, not immunologically pure is indicated by the presence of more than one precipitin band in the agar double-diffusion plates.

Despite the large differences in amino acid composition and in catalytic and immunological properties of the chicken supernatant and mitochondrial malic dehydrogenases, it appears likely that the enzymes have some structural features in common, since both enzymes catalyze the same chemical reaction. Indirect evidence that this is the case comes from our recent finding that it is possible, using procedures published elsewhere (Chilson *et al.*, 1965, 1966), to produce an enzymatically active hybrid between the chicken mitochondrial and supernatant enzymes.

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