Purification and Properties of Two Types of Diphosphopyridine Nucleotide-linked Glycerol 3-Phosphate Dehydrogenases from Chicken Breast Muscle and Chicken Liver*

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SUMMARY

We have purified and compared two different L-glycerol 3-phosphate:DPN-linked oxidoreductases (EC 1.1.1.8) from chicken, one from liver and the second from breast muscle. The crystalline enzyme from chicken breast muscle appears homogeneous by all tests applied. The purified glycerol 3-phosphate dehydrogenase from chicken liver appears homogeneous by electrophoresis and sedimentation ultracentrifugation; however, it is not homogeneous as an antigen as determined by immunodiffusion.

Both enzymes have molecular weights between 60,000 and 65,000 and appear to be composed of two identical subunits. The amino acid compositions are similar, with only glycine, glutamic acid plus glutamine, and arginine differing by more than 3 residues per subunit. There are 2 tryptophan residues per subunit in each enzyme.

These enzymes are markedly different by other criteria. Antiserum, when added to each of these proteins, cross-reacts very weakly with the heterologous antigen. Electrophoresis at pH 8.6 shows that glycerol 3-phosphate dehydrogenase from chicken liver is negatively charged, whereas the corresponding breast muscle enzyme is positively charged.

Rather striking differences are found in a comparison of the catalytic properties of glycerol 3-phosphate dehydrogenase from chicken liver and chicken breast muscle. The binding constants for oxidized and reduced diphosphopyridine nucleotide and the $K_m$ values for dihydroxyacetone phosphate and glycerol 3-phosphate are, respectively, 5-, 25-, and 100-fold lower for the liver enzyme than for the muscle enzyme. If the catalytic properties in vitro are present under conditions in vivo, glycerol 3-phosphate dehydrogenase from chicken muscle would be essentially unable to catalyze the oxidation of glycerol 3-phosphate.

Peptide maps from tryptic digests of these two enzymes show little, if any, similarity, indicating that glycerol 3-phosphate dehydrogenases from chicken liver and chicken breast muscle are products of separate genes rather than tissue-specific modifications of a single gene product.

We propose different physiological roles for the two glycerol 3-phosphate dehydrogenases of chicken. The form present in skeletal muscle may operate in concert with muscle type lactate dehydrogenase to regenerate diphosphopyridine nucleotide during anaerobic glycolysis. The form which predominates in chicken liver is likely to function in triglyceride and phospholipid metabolism. Our data are consistent with, but do not prove, these foregoing hypotheses.

The presence in one organism of more than one enzyme to catalyze a particular reaction has been demonstrated for many enzymes in many organisms (1). Commonly, these multimolecular forms of enzymes occur in different tissues, in different cell compartments, or at different times in the development of an organism. From an evolutionary point of view, it seems likely that each enzyme form, or the gene which codes for it, must have a particular selective importance which is different from the other forms which catalyze the same reaction. On the basis of catalytic properties, substrate specificities, and tissue distribution, different selective roles have been suggested for the multimolecular forms of aldolase (2), lactate dehydrogenase (3, 4), pyruvate kinase (5), and a number of other enzymes.

Several multimolecular enzyme systems have been studied in the chicken (6-11). In addition to these systems, glycerol-3-P dehydrogenase has been shown to exist in two forms with relative amounts which vary depending upon the tissue studied (12). We have purified both of these molecular forms and have studied their physical and chemical properties in an attempt to determine their physiological roles and thus their respective selective importance.

EXPERIMENTAL PROCEDURE

Materials—Dihydroxyacetone phosphate dicyclohexylammonium salt dimethylketal was obtained from Calbiochem. $\alpha$-Glycerol phosphate was obtained from Sigma. The enzymes, DPN$^+$ and DPNH, were purchased from P-L Biochemicals. DEAE-cellulose (DE-11) and phosphocellulose (P-11) were obtained from Whatman. Twice recrystallized, lyophi-
lized bovine trypsin, lot TRL-6JB, was purchased from Worthington Biochemical Corporation. Before use, it was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone as described by Carpenter (13) in order to inhibit chymotryptic activity.

**Determination of Glycerol-3-P Dehydrogenase Activity**—Glycerol-3-P dehydrogenase activity was routinely measured at room temperature in a Zeiss model PMQ II spectrophotometer by following the decrease in optical density at 340 nm through a 1-cm path in a 3.0-ml reaction mixture. The reaction mixture contained 50 mM Tris-Cl (pH 8.0), 0.5 mM dehydroxyacetone phosphate, and 0.1 mM DPNH. The reaction was initiated by the addition of enzyme (0.01 to 0.5 ml) sufficient to cause an optical density decrease of 0.100 to 0.200 per min. The amount of enzyme required to catalyze a 1.00 optical density change per min is defined as 1 unit of activity and is equivalent to 0.48 pmole of DPNH oxidized per min. The $K_m$ for dehydroxyacetone phosphate was determined under the above conditions with a constant amount of purified enzyme and varying amounts of dihydroxyacetone phosphate.

Two procedures were used to prepare dihydroxyacetone phosphate. For kinetic studies, the diacetylxyloaminic acid obtained, dimethylketal monohydrate, was treated with ion exchange resin, hydrolyzed, neutralized, and stored according to the published procedure (15). The crystalline aldolase used for this procedure was obtained in gram quantities during the purification of glycerol-3-P dehydrogenase (see "Purification and Crystallization of Glycerol-3-P Dehydrogenase from Chicken Breast Muscle"). The concentration of dihydroxyacetone phosphate in stock solutions was determined spectrophotometrically by measuring the stoichiometric amounts of DPNH oxidized at the time when the reaction went to completion in the presence of excess enzyme.

The $K_m$ determinations for glycerol-3-P and DPN$^+$ were determined at room temperature in a Cary model 15 spectrophotometer using the sensitive slide wire and recording the initial rate of increase in optical density at 340 nm. The 3.0-ml reaction mixtures contained 50 mM Tris-Cl (pH 9.0), DPN$^+$, and glycerol-3-P in appropriate amounts. The concentrations of glycerol-3-P solutions were calculated assuming that the glyceraldehyde-3-P was a racemic mixture containing 95% of the $\alpha$ isomer as indicated by the supplier.

**Fluorescence Studies**—The binding constants for DPN$^+$ and DPNH and the stoichiometry of DPNH binding to the glycerol-3-P dehydrogenases were determined fluorometrically by the method of Velick (16) in a fluorimeter designed and constructed by Mr. Burkov Bablousian.

**Protein Determinations**—During purification steps, protein concentrations were estimated by the method of Warburg and Christian (17). For the calculation of extinction coefficients and specific activities of the purified enzymes, protein determinations were made using the Miller modification (18) of the Lowry method. Bovine serum albumin was used as a standard.

**Extinction Coefficients**—The extinction coefficients for both enzymes were determined by correlating the optical density at 280 nm with the protein concentration determined by the Miller method. A molecular weight of 60,000 was assumed for these calculations. The extinction coefficient is expressed as the optical density of a 1 mM enzyme solution at 280 nm with a 1-cm light path.

**Amino Acid Analysis**—The amino acid composition, with the exception of tryptophan and cysteine, was determined by the method of Spackman, Stein, and Moore (19) using a Beckman model 120C amino acid analyzer. Duplicate hydrolyses of 24, 48, and 72 hours were carried out in 6 N HCl at 110$°$C. The results were averaged, or where degradation or slow release was apparent, extrapolated values were used. Cysteine was determined as cysteic acid in the above manner after a 2-hour performic acid oxidation (20). Tryptophan determinations were made spectrophotometrically in 6 M guanidine hydrochloride according to the procedure of Edelhoch (21).

**Tryptic Digestion and Peptide Mapping**—The following steps were carried out simultaneously under identical conditions on 10-mg samples of purified glycerol-3-P dehydrogenase from chicken liver and chicken breast muscle. The free sulfhydryl groups of each sample were carboxymethylated with a 10-fold excess of iodoacetate in 8 M urea for 2 hours at room temperature. The free and excess iodoacetate were removed by dialysis against 10$^-4$ M HCl. The samples were treated with trypsin for 5 hours at 37$°$C as described by Harris and Perham (22). The procedures used for peptide mapping were also those described by Harris and Perham. The peptides which were acidic on electrophoresis at pH 6.5 were cut out, sewn on fresh sheets of Whatman No. 3MM paper, and chromatographed side by side. The same procedure was used for the "basic" peptides. "Neutral" peptides were separated by electrophoresis at pH 3.5 on fresh paper and then chromatographed as described above. In all cases the chromatography step was carried out in triplicate and the peptides were subsequently detected by ninhydrin, the Sakaguchi stain for arginine (23), or the Pauly stain for histidine (23).

**Electrophoresis**—During the purification of glycerol-3-P dehydrogenase from chicken liver and chicken breast muscle, starch gel electrophoresis was used routinely. Agar (1%) and hydrolyzed potato starch (1.5%) in 0.02 M sodium barbital buffer, pH 8.6, were boiled, and the homogeneous solution was poured onto lantern slide plates (20 ml per plate). After the gels formed, they were covered with Saran Wrap and stored for up to 1 week at 4$°$C. Samples were introduced into slits in the gel and electrophoresis was carried out for 45 min at 35 ma. The electrode wells contained 0.06 M barbital buffer. Staining for glycerol-3-P dehydrogenase activity was performed exactly as described for lactate dehydrogenase (24) except that 2 M DL-glycerol-3-P was used instead of 2 M DL-lactate. Acrylamide disc electrophoresis and protein staining were performed as described by Davis (25).

**Ultracentrifugal Analysis**—Ultracentrifugal equilibrium (28) and sedimentation velocity studies were performed in a Beckman model E ultracentrifuge. Protein samples were dialyzed against 100 mM sodium phosphate (pH 6.2), 2 mM EDTA, and 100 mM 2-mercaptoethanol before being used for ultracentrifugal studies.

**Immunological Procedures**—Rabbit antisera for chicken liver and chicken breast muscle glycerol-3-P dehydrogenases were obtained by injecting 1 ml of a solution containing 3 to 5 mg of purified enzyme with an equal amount of Freund's adjuvant and injecting this into adult rabbits (27). The antisera obtained with this method were used directly for immunodiffusion studies (28).
Purification and Crystallization of Glycerol-3-P Dehydrogenase from Chicken Breast Muscle—Unless otherwise stated the following purification procedure was carried out at 6°C. All centrifugations were done in a Servall centrifuge at 9,000 rpm using a GSA head or at 18,000 rpm using an SS-34 head. The pH measurements were made at 6°C. Dialysis tubing was boiled at least twice in 0.05 M EDTA before use.

Extraction—Approximately 6 kg of chicken breast muscle, obtained from 30 pounds of chicken breasts, was passed through an electric meat grinder and then extracted for 1 hour with occasional stirring in 3 liters of cold distilled water per kg of muscle. (All solutions used in this purification contain 1 mM EDTA and 1 mM 2-mercaptoethanol.) Large tissue fragments are removed by straining the solution through two layers of cheesecloth. The remaining cellular debris was removed by centrifugation at 9000 rpm for 30 min. The supernatant is poured through a funnel containing a plug of glass wool which removes much of the fat floating on the surface.

Ammonium Sulfate Fractionation—To the above crude extract, 431 g of ammonium sulfate per liter of extract (65% saturation) were added over a 1-hour period with continuous stirring. The resulting precipitate is then collected by centrifugation at 9,000 rpm for 15 min. The supernatant (which contains less than 10% of the glycerol-3-P dehydrogenase activity) can be discarded. Neutralization of this supernatant fraction yields a gelatinous precipitate of glyceroldehyde-3-P dehydrogenase of greater than 50% purity. The extraction solution is added to the 65% precipitate by stirring until the density drops to 1.135 (about 45% saturation). Undissolved and denatured proteins are removed by centrifugation at 9,000 rpm for 10 min. The resulting supernatant is then dialyzed overnight against 10 volumes of 54% saturated ammonium sulfate (343 g per liter). Centrifugation of the dialyzed solution for 10 min at 9,000 rpm removes much of the precipitated protein; however, a copious fine precipitate of crystalline aldolase remains suspended and can best be removed by centrifugation at 18,000 rpm for at least an hour. The aldolase thus obtained is recrystallized several times and used in the preparation of substrate as described earlier.

Heat Step—The supernatant obtained after the removal of aldolase is heated to 70°C with constant stirring. After 5 min, the solution is cooled rapidly in an ice bath. The denatured proteins are removed by centrifugation at 9000 rpm for 10 min. A 10-fold purification is often obtained at this step.

DEAE-cellulose Column Chromatography—The supernatant protein from the previous step is precipitated by the addition of 60 g of solid ammonium sulfate per liter of solution. The precipitate is collected by centrifugation at 9000 rpm for 10 min and then dissolved in a minimum amount of 5 mM Tris-HCl, pH 7.6. The redissolved protein is dialyzed twice against 40 volumes of 5 mM Tris-HCl, pH 7.6. The dialyzed solution, containing between 40 and 60% of the initial activity, is applied to a 2.6-liter DEAE-cellulose column (4.5 x 124 cm), and this is equilibrated with the above buffer. The amount of protein added to the column should not exceed 3 g per liter of packed volume. A 4-liter linear gradient of NaCl (0 to 0.3 M) in an equilibration buffer is begun shortly before the breakthrough volume has passed. Glycerol-3-P dehydrogenase will elute at the beginning of the gradient. The pH of this step is quite critical because if it is slightly low, the enzyme will not be retained by the column; if it is slightly high, the yield of enzyme is drastically reduced.

Phosphocellulose Column Chromatography—The active fractions from the preceding step are of greater than 90% purity. The remaining contaminants are removed, and the glycerol-3-P dehydrogenase is concentrated by adding the pooled fractions from the DEAE-cellulose column directly to a 400-ml phosphocellulose column (2.8 x 75 cm) equilibrated with 5 mM sodium phosphate at pH 6.2. Immediately after the sample has been applied, a 2-liter linear NaCl gradient (0 to 1.0 M) is started. The resulting eluted enzyme is pure.

Crystallization—Needle-like crystals of chicken breast muscle glycerol-3-P dehydrogenase form after dialysis of the active fractions against the above sodium phosphate buffer saturated with ammonium sulfate.

Purification of Glycerol-3-P Dehydrogenase from Chicken Liver—The temperature, centrifugation conditions, pH measurements, and dialysis tubing treatment are the same as those described for the purification of chicken muscle glycerol-3-P dehydrogenase. All buffers and ammonium sulfate solutions used in this purification contain 1 mM EDTA and 10 mM 2-mercaptoethanol.

Extraction—Homogenized in two batches for 1 min in a 4-liter blender are 6.3 liters of 48% saturated ammonium sulfate (300 g per liter) and 1.36 kg of thawed chicken livers. Cellular debris is removed by centrifugation for 40 min at 9000 rpm. The supernatant is decanted through a funnel with a glass wool plug to remove fat floating on the surface. The precipitate does not pack well, and therefore care must be taken to prevent it from contaminating the supernatant which contains the glycerol-3-P dehydrogenase activity.

Ammonium Sulfate Fractionation—By the slow addition of ammonium sulfate (180 g per liter), the concentration is increased to about 65% saturation. The precipitate, which contains the enzyme, is collected by a 30-min centrifugation at 9000 rpm. The supernatant is discarded and the precipitate is suspended in a minimum amount of 5 mM Tris-HCl buffer, pH 7.6. Material which remains insoluble at a density of 1.135 (about 45% saturation) is removed by centrifugation.

DEAE-cellulose Column Chromatography—The preceding supernatant is dialyzed twice against 15 volumes of 5 mM Tris-HCl at pH 7.2 over an 18-hour period. The precipitate which forms after dialysis is removed by centrifugation at 9000 rpm for 15 min. The dialyzed solution is applied to a 2.6-liter DEAE-cellulose column (4.5 x 124 cm) equilibrated with the above buffer. Since the chicken liver glycerol-3-P dehydrogenase binds relatively tightly to DEAE-cellulose, as much as 40 g of protein may be added per liter of packed column volume. Immediately after the sample is applied, a 10-liter linear gradient (0 to 0.4 M NaCl) in 5 mM Tris-HCl, pH 7.2, is started.

Phosphocellulose Column Chromatography—Active fractions from the preceding column are added directly to a 270-ml phosphocellulose column (2.8 x 43 cm) equilibrated with 5 mM sodium phosphate buffer, pH 6.5. The enzyme is eluted with a 1-liter linear gradient (0 to 0.25 M ammonium sulfate) in the preceding buffer.

Sephadex G-100 Column Chromatography—The active fractions from the preceding step are precipitated by dialysis against saturated ammonium sulfate. The precipitate is collected by centrifugation at 9000 rpm for 10 min and then dissolved in a minimum amount of sodium phosphate buffer, pH 6.5, containing...
0.25 M ammonium sulfate. This sample is applied to a 500-ml Sephadex G-100 column (2.8 × 78 cm) equilibrated with the preceding buffer.

Heat Step—To the above active fractions solid DPNH and neutralized N-glycerol-3-P are added to concentrations of 0.1 and 30 mM, respectively. Precipitated proteins are removed by centrifugation. Optical estimation of the purification at this step is not possible because of the absorbance of the pyridine nucleotide; however, there is no loss of glycerol-3-P dehydrogenase activity.

Second Sephadex G-100 Column—The supernatant from the above heat step is precipitated by dialysis against saturated ammonium sulfate and the procedure for the previous Sephadex G-100 step is repeated. The fractions with constant specific activity, representing about 60% of the added enzyme, are pooled. The enzyme at this stage appears homogeneous by most criteria but has not been crystallized.

RESULTS

Isolation of Glycerol-3-P Dehydrogenase from Chicken Breast Muscle and Chicken Liver—Quantitative summaries for the purification of glycerol-3-P dehydrogenase from chicken breast muscle and chicken liver are shown in Tables I and II, respectively. These purifications have been repeated successfully several times. Since the electrophoretic properties of both enzymes remain constant throughout purification, it seems unlikely that any gross modification of either enzyme occurs during the isolation procedure. Fig. 1 shows the electrophoretic behavior of the two different glycerol-3-P dehydrogenases in crude extracts from chicken liver and as purified enzymes.

During purification both enzymes are stabilized by the presence of 2-mercaptoethanol. DPNH also stabilizes these enzymes to thermal denaturation, as mentioned by Rouslin (12), particularly in solutions of low ionic strength. The use of DPNH for stabilization is only feasible in the later stages of purification when small volumes are involved.

Criteria of Purity—Both enzymes appear homogeneous on starch-agar and acrylamide gel electrophoresis. Impurities of 5% should have been detected by the procedures used. Analytical ultracentrifugation revealed a single symmetrical peak for both enzymes (Fig. 2). Ouchterlony immunodiffusion of crystalline chicken muscle glycerol-3-P dehydrogenase against its antisera showed a single precipitin band. Initially, this band...
**FIG. 2.** Schlieren peaks of sedimentation velocity ultracentrifugal analysis of glycerol-3-P dehydrogenase from chicken breast muscle (upper trace) and chicken liver (lower trace). The interval between photographs was 8 min and the rotor speed was 58,000 rpm.

**TABLE III**  
Comparison of apparent binding constants of chicken muscle and liver glycerol-3-P dehydrogenases for DPN, DPNH, glycerol 3-phosphate, and dihydroxyacetone phosphate

<table>
<thead>
<tr>
<th>Apparent binding constants</th>
<th>Glycerol-3-P dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chicken liver</td>
</tr>
<tr>
<td>$K_m$ dihydroxyacetone phosphate</td>
<td>0.043</td>
</tr>
<tr>
<td>$K_m$ glycerol-3-P</td>
<td>0.08-0.12</td>
</tr>
<tr>
<td>$K_m$ DPN</td>
<td>0.02-0.04</td>
</tr>
<tr>
<td>$K_m$ DPNH</td>
<td>0.02</td>
</tr>
<tr>
<td>$K_m$ DPN$^+$</td>
<td>$\sim0.0014$</td>
</tr>
</tbody>
</table>

*An approximation based on double reciprocal plots of fluorescence titration experiments. See Fig. 4.*

was observed for the chicken liver enzyme, but after the enzyme had been standing a day, a second faint precipitin band appeared. Based on the above criteria, the crystalline glycerol-3-P dehydrogenase from chicken muscle is homogeneous and the glycerol-3-P dehydrogenase from chicken liver contains less than 5% contamination.

**Extinction Coefficients and Specific Activities**—The extinction coefficient at 280 nm (optical density of a 1 mM solution) determined for two different preparations of glycerol-3-P dehydrogenase from chicken liver were 35.2 and 36.5. The corresponding values for the chicken muscle enzyme were 27.5 and 29.1. An optical density at 280 nm of 1.0 is equivalent to 2.15 mg per ml of chicken muscle glycerol-3-P dehydrogenase and 1.70 mg per ml of chicken liver glycerol-3-P dehydrogenase. The maximum specific activities obtained with the standard assay system

**FIG. 3.** Determination of DPN$^+$-binding constants of glycerol-3-P dehydrogenase from chicken breast muscle (A) and chicken liver (B). Enzyme samples were excited with 288 nm light and the emission at 340 nm was measured after successive additions of DPN$^+$. Figure are double reciprocal plots of the decrease in protein fluorescence versus the DPN$^+$ concentration.
were 303 units per mg and 185 units per mg, respectively, for the muscle and liver enzyme.

Catalytic Properties—The observed $K_m$ values for the two glycerol-3-P dehydrogenases are listed in Table III. In every case these values are lower for the chicken liver enzyme.

$DPN^+$- and $DPNH$-binding Properties—Figs. 3 and 4 show the results of titrating each enzyme with $DPN^+$ or $DPNH$ where the quenching of protein fluorescence was followed. The $K_s$ values calculated from these data are listed in Table III. The $K_s$ values calculated for $DPN^+$ compared closely with the corresponding $K_m$ values observed for the respective enzyme. The values of $K_s$ calculated for $DPNH$ are only approximations derived from double reciprocal plots. These numbers are probably slightly high, since the concentrations of $DPNH$ in these experiments were in the range of 5- to 20-fold in excess over the available binding sites. Despite this difficulty, it still appears that $DPNH$ is also bound more tightly to chicken liver glycerol-3-P dehydrogenase.

The stoichiometry of $DPNH$ binding to the chicken muscle enzyme was very close to 2 molecules per enzyme molecule of molecular weight 60,000. The greater lability of the chicken liver enzyme made this stoichiometry determination more difficult. It was assumed that a decrease in specific activity of the sample used reflected a corresponding loss in ability to bind $DPNH$. Using this correction, the observed stoichiometry was 1.7 molecules of $DPNH$ bound per enzyme molecule of molecular weight 60,000.

When the $DPNH$-enzyme binary complexes were excited with light at 340 nm, $DPNH$ fluorescence enhancements of 2.5 and
Fig. 6. Comparison of "acidic" tryptic peptides of glycerol-3-P dehydrogenase from chicken breast muscle and chicken liver. Faint ninhydrin spots are indicated by ——. Y, yellow ninhydrin spots; O, orange ninhydrin spots. All other spots are pink. Peptides showing a positive reaction for arginine or histidine are indicated.

Fig. 7. Comparison of "neutral" peptides of glycerol-3-P dehydrogenase from chicken breast muscle and chicken liver. Faint ninhydrin spots are indicated by ——. Y, yellow ninhydrin spots; O, orange spots. All other spots are pink. Peptides showing a positive reaction for arginine or histidine are indicated. The three spots on the left side of each map are probably free amino acids which represent systemic contaminants rather than specific components of the tryptic digests.

3.6 were observed at 460 nm for the muscle and liver enzymes, respectively. Stoichiometric titrations under these conditions yielded the same values given above.

Amino Acid Analysis—The amino acid composition for the two glycerol-3-P dehydrogenases from chicken are shown in Table IV. The two sets of data are remarkably similar with only arginine, glutamic acid and glutamine, and alanine differing by more than 3 residues per subunit. The largest relative and absolute difference is found with arginine, where there are 6 residues per subunit in the muscle enzyme and 12 in the liver enzyme.

Peptide Maps—Figs. 5, 6, and 7 compare the peptide maps obtained from tryptic digests of glycerol-3-P dehydrogenase from chicken liver and chicken muscle. Peptides showing a positive reaction for arginine or histidine are indicated. The maps show little if any similarity. The number of ninhydrin spots slightly exceeds the expected number, which may indicate partial hydrolysis of some peptides or a slight underestimation of the lysine and arginine composition. The number of histidine-containing peptides is consistent with the amino acid analysis for both enzymes. There were about 11 arginine-containing peptides for both enzymes. This would be the number expected for the chicken liver enzyme but it is greater than the number expected for chicken muscle glycerol-3-P dehydrogenase. How-
ever, some of the arginine peptides of the chicken breast muscle were found to be quite minor components.1

**Ultracentrifugal Analysis**—Simultaneous sedimentation velocity studies such as that shown in Fig. 2 indicate that these enzymes have virtually identical S values. The $s_{20,w}$ values from two different simultaneous runs are listed in Table V. Also shown in Table V are the molecular weight values obtained from equilibrium ultracentrifugation and gel filtration studies. Both methods suggest molecular weights in the 60,000 to 67,000 range.

**Electrophoretic Properties**—Fig. 1, as mentioned previously, shows the electrophoretic mobility of the two glycerol-3-P dehydrogenases in purified form and in crude extracts. The chicken muscle form has a net positive charge near neutrality, whereas the chicken liver form is negatively charged.

**Immunological Cross-reaction**—Fig. 8 shows the immunodiffusion of the purified chicken glycerol-3-P dehydrogenases against their homologous and heterologous antisera. In both cases, the homologous reaction is strong and the heterologous reaction is very weak or nonexistent.

**DISCUSSION**

Rouslin (12) first suggested from catalytic and chemical observations with crude extracts that multiple forms of glycerol-3-P dehydrogenase existed in chicken and that these forms had different tissue distributions. The two DPN-linked glycerol-3-P dehydrogenases we have purified and studied correspond to the forms Rouslin found. Our studies on the purified enzymes confirm and quantitate some of the differences found in crude extracts. Also, we have demonstrated additional contrasting physical and chemical properties.

The most interesting differences between the purified glycerol-3-P dehydrogenases from chicken liver and chicken breast muscle are in their catalytic parameters. The Michaelis constants for dihydroxyacetone phosphate, glycerol-3-P, and DPN and the binding constants for DPN and DPNH are lower with the chicken liver enzymes. These differences are not nearly as large for the dihydroxyacetone phosphate and DPNH as they are for glycerol-3-P and DPN. Since the $K_m$ values of glycerol-3-P dehydrogenase from rabbit and rat muscle vary considerably depending upon the buffer used in the assay (29, 31), the observed $K_m$ values in Table III may not represent physiological properties of the chicken glycerol-3-P dehydrogenases from chicken. However, the $K_m$ value of chicken muscle glycerol-3-P dehydrogenase for glycerol-3-P is so high compared with the physiological levels of this substrate, that it suggests that the reaction catalyzed by this enzyme under these conditions is essentially irreversible. Studies on DPN-linked cytoplasmic metabolites in rat muscle during contraction and recovery (39) indicate that glycerol-3-P formed during contraction is converted back to dihydroxyacetone phosphate during recovery via the flavin-linked mitochondrial pathway, rather than via the reversal of the DPN-linked reaction. Thus under conditions in muscle in vivo, it also appears that glycerol-3-P dehydrogenase catalyzes only the reduction of dihydroxyacetone phosphate.

The reported catalytic properties of crystalline muscle glycerol-3-P dehydrogenase from rat (29) and rabbit (30, 31) are similar to those we observed with the chicken liver enzyme, and contrast unexpectedly with the catalytic properties of the enzyme from chicken muscle, a homologous tissue. It appears from electrophoresis of crude extracts from rat liver, muscle, heart, mammary gland, and adipose tissue that there is one predominant electrophoretic form in all these tissues. It was only in the liver that there was a very slight indication of the possibility of a second form. These observations would suggest that in the rat a diversification of glycerol-3-P dehydrogenase has not occurred or has been suppressed during evolution, or possibly that two forms have identical electrophoretic mobility.

Recent reports have shown that the molecular weight of glycerol-3-P dehydrogenase from honey bee (30, 34), rat muscle (29), and rabbit muscle (31) is between 60,000 and 67,000 as opposed to the earlier estimate of 76,000 for the rabbit muscle enzyme (35). Our studies with these dehydrogenases from chicken are in agreement with the more recent estimates of their molecular weight. The $s_{20,w}$ values we observed ranged from 4.2 to 4.6 depending upon the enzyme concentration. These values are in the range of maltose dehydrogenase, a protein of similar molecular weight. We did not experience the dissociation noted by Fondy et al. (29) for the rat muscle enzyme.

Genetic studies on glycerol-3-P dehydrogenase from *Drosophila* (36) show clearly that the enzyme is a dimer normally composed of two identical subunits. The electrophoresis gel with three bands for enzyme activity which we observed with one chicken muscle preparation indicates that the glycerol-3-P dehydrogenase from chicken is also a dimeric protein. Further confirmation of the dimeric structure comes from the fact that there are two DPNH binding sites per molecule of 60,000 molecular weight. This value has also been obtained for the rabbit muscle enzyme assuming a higher molecular weight than we used (37).

Our amino acid calculations are based on the belief there are two identical subunits per enzyme molecule. The amino acid analysis shows very similar compositions for glycerol-3-P dehydrogenase from chicken breast muscle and chicken liver, and these in turn are quite similar to the values reported for the enzyme from rat and rabbit muscle. The only difference readily apparent is that chicken, rat, and rabbit muscle glycerol-3-P dehydrogenases have nearly equal amounts of arginine and histidine, whereas the chicken liver enzyme has relatively more arginine and less histidine.

Crystalline rabbit muscle glycerol-3-P dehydrogenase prepared by ammonium sulfate fractionation appears to have a tightly bound nonprotein component identified by Ankel, Bücher, and Czok (38) as adenosine diphosphoribose. Since we used frequent dialyses and column steps in our purification, it is quite likely that any such component, if present, would have been removed during enzyme purification. We did not notice particularly low $A_{260}/A_{280}$ ratios which might be indicative of such a nonprotein component.

From these studies we would like to be able to assign different physiological roles for the two glycerol-3-P dehydrogenases and thus justify their evolutionary existence. There are two important roles which can be served by glycerol-3-P dehydrogenase. One involves the continued operation of glycolysis. DPNH formed in the glyceraldehyde-3-P dehydrogenase reaction must be coupled to an enzyme which reoxidizes it to DPN. Several enzymes, such as lactate dehydrogenase in vertebrate muscle and alcohol dehydrogenase in yeast, have evolved to perform this function. In the flight muscles of insects, it is glycerol-3-P dehydrogenase which is coupled to glyceraldehyde-3-P dehydrogenase.

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1 More recent analysis of the muscle enzyme showed only 7 to 8 arginine peptides.

2 H. B. White, unpublished observations.
dihydrogenase (39). A second role is the formation of glycerol-3-P which is a precursor for triglycerides and phospholipids. Although it is conceivable that both functions could be served by a single enzyme, it would seem more likely that in higher organisms with division of labor among various organs and tissues, that these functions would reside in different enzymes under independent genetic and metabolic control.

Our studies do not permit the unequivocal assignment of these separate roles to the two glycerol-3-P dehydrogenases from chicken; however, several lines of evidence suggest that the enzyme from chicken muscle can be associated with a specific physiological function. It was shown by Dawson and Kaplan (40) that both glycerol-3-P dehydrogenase and muscle-type lactate dehydrogenase appear simultaneously in chicken breast muscle during the first day after hatching and that the level of these enzymes increases steadily for a month. They also showed that in birds which have high proportions of heart type lactate dehydrogenase, glycerol-3-P dehydrogenase is either absent or present in very low amounts. This striking correlation between muscle-type lactate dehydrogenase and glycerol-3-P dehydrogenase in the ontogeny and phylogeny of avian flight muscle suggests very strongly that these two enzymes have a very similar and closely linked physiological role, the maintenance of relatively anaerobic glycolysis in muscle tissue.

Evidence for the involvement of glycerol-3-P dehydrogenase from chicken liver in triglyceride or phospholipid biosynthesis is at best circumstantial. The strongest evidence is that liver is the major site of lipid synthesis in chicken (41, 42) and the chicken liver glycerol-3-P dehydrogenase is apparently the only enzyme present which can supply glycerol-3-P for lipid biosynthesis. The acyl dihydroxyacetone phosphate pathway to phospholipid synthesis (43, 44) has not been investigated. One might expect that if liver glycerol-3-P dehydrogenases were involved in lipid synthesis, it would be a metabolically regulated branch point enzyme, and that TPN would be a cofactor (45, 46). These possibilities have as yet not been investigated.

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