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Isolation and Characterization of Tissue-specific Isozymes of Glucosephosphate Isomerase from Catfish and Conger*

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In teleosts glucosephosphate isomerase exists as two tissue-specific isozymes. Most tissues contain the more acidic liver-type isozyme, while white muscle contains the more basic isozyme; and a few tissues contain both the liver- and muscle-type isozymes as well as a hybrid. The isozymes were isolated from catfish liver and muscle and from conger muscle and shown to be homogeneous by polyacrylamide gel electrophoresis, isoelectric focusing, analytical ultracentrifugation, and rechromatography. Both isozymes are of molecular weight 132,000 ($s^{0}_{20,w}$ = 7.0 S) and composed of two subunits of M_r approximately 65,000. The muscle and liver isozymes were shown to have distinct isoelectric points (catfish liver = 6.2; muscle = 7.0) and amino acid compositions. Tryptic peptide maps, after *S*-carboxymethylation and carbamylation, revealed several distinct differences in the primary structures of the isozymes. Although the isozymes could also be distinguished on the basis of their stabilities, most of their basic catalytic properties were found to be similar. A conger was obtained which was heterozygous for the variant allele at the muscle-glucosephosphate isomerase locus. A comparison of the variant conger muscle isozyme with the wild type revealed a single altered peptide, suggesting a point mutation. The structure-function studies, as well as the genetic studies, clearly establish that the two types of isozymes are of independent genetic origin.

Glucosephosphate isomerase (EC 5.3.1.9) has been the subject of several recent studies relating to multiple electrophoretic forms of the protein. Genetically determined variant forms have been observed in mice (2, 3), rabbits (4), swine (5, 6), humans (7–9), and other species (4, 10–14). Heterozygous individuals usually exhibit three-banded glucosephosphate isomerase zymograms typical of dimeric enzymes. Although multiple forms of glucosephosphate isomerase from homozygous individuals have been observed by electrophoresis, isoelectric focusing, and ion-exchange chromatography (15–18), these multiplicities have been shown recently to result from the oxidation of sulfhydryl groups, and are not of genetic origin (15, 16). Payne *et al.* (16) examined a variety of rat and human tissues by isoelectric focusing and demonstrated that tissue-specific isozymes of glucosephosphate isomerase do not exist. Furthermore, a recent survey of over 25 species of vertebrates, invertebrates, and microorganisms revealed a single electrophoretic form of glucosephosphate isomerase in most species (19).

In marked contrast, electrophoretic studies on tissue extracts from teleostean fish (20–26) have suggested that most fish exhibit tissue-specific isozymes of glucosephosphate isomerase which appear to be the result of independent autosomal gene loci. These unique tissue-specific isozymes thus provide an

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interesting and unique system for studying structure-function relationships of this enzyme. These isozymes also raise fundamental questions regarding their physiological roles in different tissues, as well as their evolutionary origin. The isolation of these isozymes and the elucidation of the catalytic, chemical, and physical properties of these proteins were undertaken in order to establish the fundamental structural and functional properties.

MATERIALS AND METHODS

Fish—Catfish, *Ictalurus punctatus* (Rafinesque), were obtained live from Lake Texoma, Tex., while conger, *Conger conger* (L.), were captured by trawl and long-line in the English Channel off Plymouth, Devon. Tissues were removed from freshly killed fish and used immediately or stored at −20° before use.

Materials—All substrates, coupling enzymes, and coenzymes were obtained from Sigma Chemical Co. Ampholine carrier ampholytes and all equipment used in isoelectric focusing were from LKB Produkter, while supplies and equipment for polyacrylamide gel electrophoresis were from Bio-Rad. DEAE-cellulose (DE52) and CM-cellulose (CM52) were purchased from Whatman Biochemicals. Reagents for enzyme activity stains, phenazine methosulfate, and 3(4,5-dimethylthiazolyl-2)-2-5-diphenyl tetrazolium salt and protein stains, Coomassie brilliant blue and naphthol blue black were from Sigma.

Enzyme Assays—Glucosephosphate isomerase activity was assayed in the reverse direction (fructose 6-phosphate→glucose 6-phosphate) by coupling with excess glucose 6-phosphate dehydrogenase and measuring the rate of reduction of NADP at 340 nm (27). Substrate concentrations were determined enzymatically. All assays were carried out at 30° in a recording spectrophotometer, and 1 unit of enzyme is defined as the amount catalyzing the isomerization of 1.0 μmol of substrate/min at 30°. Values for Michaelis constants and maximal velocities were calculated by a weighted least squares regression analysis (28) adapted for the IBM 360-50 computer (29).

Protein Determination—Protein concentrations of fractions collected from chromatography and isoelectric focusing were estimated routinely from the absorbance at 280 nm, but more accurate measurements of pooled enzyme solutions were determined by the biuret procedure (30) using serum albumin as a standard.

Amino Acid Analyses—Amino acid analyses of acid hydrolysates were carried out on a Beckman 120C automatic amino acid analyzer (31, 32). Samples were sealed and hydrolyzed *in vacuo* at 110° for 24, 48, and 72 hours in 2 ml of 6 N HCl containing 1 μl of 2-mercaptoethanol. Values for threonine and serine were extrapolated to zero time of hydrolysis. Tryptophan was determined spectrophotometrically by the method of Edelhoch (33), and cysteine was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (34).

Ultracentrifugation—Sedimentation velocity and sedimentation equilibrium ultracentrifugation were performed with a Beckman-Spinco model E analytical ultracentrifuge equipped with RTIC temperature regulation and electronic speed control. Schlieren patterns and Rayleigh interference fringes were analyzed with a Nikon model 6C microcomparator equipped with digital x-y encoders. Sedimentation equilibrium analyses were performed by the meniscus depletion method (35).

Electrophoresis and Isoelectric Focusing—Cellulose-acetate electrophoresis was carried out on Titan IV plates (7.4 × 5.8 cm) (Helena Labs, Beaumont, Tex.) in 0.1 M Tris-barbital buffer, pH 8.9. After electrophoresis the cellulose-acetate plates were stained for glucosephosphate isomerase activity as described by Scopes (36). Vertical starch-gel electrophoresis using a pH 8.7 Tris-borate-EDTA buffer was carried out according to the method of Dando (25). Polyacrylamide gel electrophoresis was carried out in standard 7% alkaline gels (pH 8.9) according to the method of Davis (37), while gel electrophoresis in the presence of sodium dodecyl sulfate was performed following the procedure of Weber and Osborn (38). Isoelectric focusing was carried out with 2% Ampholines in sucrose density gradients as described previously (39). Fractions (1.0 ml) were collected, their pH values determined (at 0°), and immediately assayed for glucosephosphate isomerase activity.

Peptide Mapping—Peptide mapping was carried out after *S*-carboxymethylation and carbamylation as previously described (9). After dialysis the samples were digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington Biochemicals) which had been coupled to Sepharose 4B. Digestion was carried out with a 1:50 (trypsin:glucosephosphate isomerase) weight ratio at 37° in 30 mM trimethylammonium formate, pH 7.5, for 8 to 12 hours. Following digestion the matrix-bound trypsin was removed by centrifugation, and the supernatant solution was lyophilized, the residue redissolved in electrophoresis buffer, and 1 to 20 μg spotted on thin layer (20 × 20 cm; 160 μm thickness) cellulose-coated plates. Thin layer peptide mapping was carried out in a Varsol-cooled microscale mapping chamber designed to allow two comparative maps to run simultaneously (9, 39). Electrophoresis was in pyridine/acetic acid/water (1/10/89) at pH 3.5, followed by chromatography in butanol/pyridine/acetic acid/water (50/33/1/40). Peptides were located by spraying with 0.02% w/v fluorescamine dissolved in peroxide-free dioxane. Peptide maps represent composites of tracings of at least five experiments for each enzyme.

RESULTS

Tissue Distribution of Glucosephosphate Isomerase Isozymes

Fig. 1*A* shows representative zymograms of glucosephosphate isomerase from different organs of the freshwater catfish. The isomerase from liver and kidney extracts exhibited a more anodal migrating band than the enzyme from skeletal muscle. A zone of minor activity (corresponding to the more anodal isozyme of liver) was observed after prolonged staining of the muscle extracts, while a small amount of the muscle isomerase was indicated in kidney and liver extracts. Heart and brain extracts showed the presence of both muscle- and liver-type isomerases and a zone of intermediate electrophoretic mobility. The intermediate isozyme was interpreted as a heterodimer resulting from hybridization of the two types of polypeptides. This hypothesis was supported by isoelectric focusing,

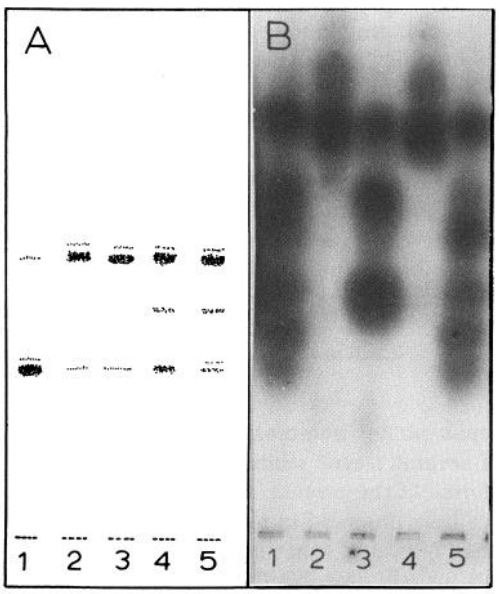

FIG. 1. Zymograms of glucosephosphate isomerase isozymes. *A*, glucosephosphate isomerases in extracts from different tissues of the catfish. Electrophoresis of catfish muscle (*1*), liver (*2*), kidney (*3*), heart (*4*), and brain (*5*) extracts was carried out on cellulose acetate plates at 200 volts and pH 8.9 for 60 min and stained for glucosephosphate isomerase activity as described under "Materials and Methods." The application sites are indicated near the bottom. The cathode was at the bottom and the anode at the top. *B*, starch gel zymogram from homozygous and heterozygous conger. Red muscle and spinal cord extracts were subjected to starch gel electrophoresis in Tris-borate-EDTA buffer, pH 8.7, at 2.5 volts/cm for 18 hours. Samples 1 and 5 were from red muscle extracts from conger heterozygous for the variant muscle glucosephosphate isomerase. Sample 2 was a spinal cord extract from the same heterozygous conger. Sample 3 was from a red muscle extract, and sample 4 was a spinal cord extract from a conger showing the common muscle glucosephosphate isomerase isozyme pattern.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analytical ultracentrifugation studies (see below).

In most conger tissues including the liver, kidney, heart, spleen, gills, brain, spinal cord, intestine, stomach, swim bladder, erythrocytes, cornea, and other eye tissues, the more anodal, liver-type, glucosephosphate isomerase isozyme predominated. Brain and spinal cord (Fig. 1*B*) also contained additional traces of glucosephosphate isomerase activities migrating slightly more anodal than the liver isozyme. Only after prolonged staining could traces of the muscle isozyme be found in any of these tissues. In white muscle the slower moving muscle-type band predominated, but traces of the liver-type isozyme were also observed occasionally. Serum and red muscle contained both muscle and liver isozymes and were unique among the conger tissues studied in exhibiting a hybrid band between the two major tissue isozymes.

A previously reported (25) more basic genetic variant of the conger muscle isozyme was observed in 5 out of 55 conger examined from the Plymouth area. A zymogram of red muscle and spinal cord extracts from a conger heterozygous for this variant allele is also shown in Fig. 1*B*. No fish that were homozygous for the variant have been found so far.

Isolation of Liver and Muscle Isozymes

In order to compare the structure-function properties of the fish isozymes at the molecular level, it was necessary to obtain the enzymes in homogeneous form. The acidic nature of the fish glucosephosphate isomerases prevented them from being bound to cellulose phosphate, and thus, it was not possible to purify them by the specific substrate elution technique developed for the more basic glucosephosphate isomerase from human (9). The following procedures were developed for the isolation of the glucosephosphate isomerase isozymes from either skeletal muscle or liver of the freshwater catfish and from the skeletal muscle of conger. The extraordinary lability of the conger liver enzyme made isolation difficult, and thus, only limited studies were possible on this isozyme.

Catfish Glucosephosphate Isomerase Purification—Catfish were killed, the tissues removed, minced, and extracted by blending for 30 s with 4 volumes of 10 mM triethanolamine buffer, pH 9.0, at 4°. The extracts were centrifuged at 20,000 × *g* for 60 min, the pellets discarded, and the supernatant solutions filtered through glass wool. After dialysis overnight against Buffer A (10 mM triethanolamine, pH 9.0, containing 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol), the extracts were subjected to chromatography on DEAE-Sephadex columns (8 × 60 cm) which had been equilibrated in Buffer A. One liter of Buffer A containing 0.01 M NaCl was pumped through the column to elute a large amount of contaminating protein. For the isolation of the muscle isozyme, a linear sodium chloride gradient of 2 liters with ionic strength ranging from 0.02 to 0.08 was applied, and the isozyme was eluted in a sharp peak at an ionic strength of 0.04. When the liver extract was chromatographed, it was necessary to employ a linear gradient of higher ionic strength (0.01 to 0.15) since glucosephosphate isomerase activity from liver eluted only at a higher ionic strength (0.11). This higher affinity of the liver isozyme for the anion exchanger points to its more acidic properties.

Fractions containing glucosephosphate isomerase activity were pooled and dialyzed against Buffer A and rechromatographed on DEAE-Sephadex columns which were developed with linear salt gradients, as shown in Fig. 2. Again, the

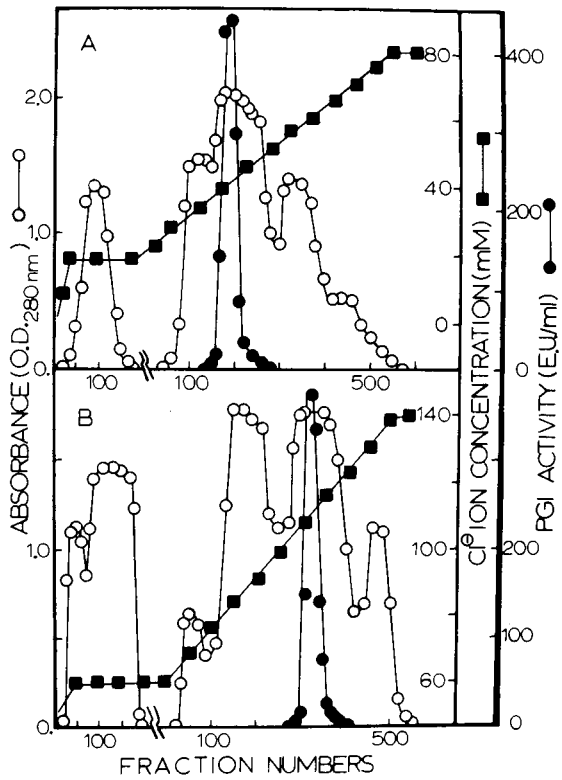

FIG. 2. Chromatography of liver and muscle isozymes on DEAE-Sephadex. *A*, partially purified catfish muscle glucosephosphate isomerase (2.8 g of protein in a volume of 50 ml; specific activity = 13 units/mg) was applied to a column, 3 × 100 cm, of DEAE-Sephadex as described in the text. The column was eluted at a flow rate of 50 ml/hour with 20 mM NaCl, 10 mM triethanolamine, pH 9.2. A shallow linear NaCl gradient (1 liter of 20 mM NaCl to 1 liter of 80 mM NaCl) was applied to elute the enzyme. *B*, partially purified catfish liver glucosephosphate isomerase (287 mg in a volume of 10 ml; specific activity = 43 units/mg) was applied to a column, 3 × 100 cm, of DEAE-Sephadex as described above. The column was washed with 60 mM salt before establishing the linear gradient (60 to 140 mM NaCl). Glucosephosphate isomerase (*PGI*) activities (●), protein concentrations (○), and chloride concentrations (■) were determined as described under "Materials and Methods."

different chromatographic properties of the two isozymes were apparent. After the second DEAE-chromatography, the enzymes exhibited average specific activities of approximately 200 units/mg and were approximately 45% pure. Some fractions were judged to be 90% pure. Over-all recoveries of isomerase activity at this stage in excess of 85% were common.

Conger Muscle Glucosephosphate Isomerase Purification—Conger white muscle was homogenized in 5 mM Tris, pH 8.0 (Buffer B), at 25°. The homogenate was heated at 44° for 30 min and the denatured protein was removed by filtration. All subsequent chromatography was carried out at 4°. The resulting extract chromatographed on a column of CM-cellulose (2.5 × 50 cm) which had been equilibrated with 5 mM phosphate buffer at pH 6.8. The glucosephosphate isomerase was eluted with a 1-liter linear sodium phosphate gradient at pH 6.8 (5 to 100 mM). Fractions containing the isomerase activity were collected, pooled, and dialyzed against Buffer B before being applied to a column (2.5 × 50 cm) of DEAE-cellulose equilibrated with Buffer B. A 600-ml linear Tris gradient at pH 8.0 (5 to 100 mM) was applied to the column, and the enzyme was then eluted with 0.2 M Tris, pH 8.0. The active fractions were combined and applied to a DEAE-cellulose column (2.5 × 50 cm) in 5 mM Tris, pH 8.2. Elution was with a 1-liter linear gradient of Tris, pH 8.0 (5 to 400 mM).

Homogeneity Studies and Physical Properties

Isoelectric Focusing and Electrophoresis—The glucosephosphate isomerase isozymes were purified further by isoelectric focusing, as shown in Fig. 3. Electrofocusing of the catfish muscle isozyme revealed a major component of glucosephosphate isomerase activity with an isoelectric pH of 7.0 and an active minor component at pH 6.6. Isoelectric focusing of liver glucosephosphate isomerase revealed a major component at pH 6.2 and a minor component at a pH of 5.6. Conger muscle glucosephosphate isomerase electrofocused with an apparent isoelectric pH of 6.4 with two minor components with isomerase activity at pH 6.6 and 6.2. These minor fractions seem to be "pseudoisozymes" (15) caused by sulfhydryl oxidation of the native enzyme, since addition of 2-mercaptoethanol or dithiothreitol greatly reduced the amounts of these minor components.

After isoelectric focusing, the isozymes from catfish muscle and liver and conger muscle were obtained with specific activities of approximately 400 to 450 units/mg and were judged to be homogeneous by a variety of criteria. Rechromatography on DEAE-cellulose, Sephadex G-200, or reisoelectric focusing did not increase the specific activity of the isozymes further. Table I summarizes the results of typical purifications of glucosephosphate isomerase from catfish muscle and liver.

Both the catfish liver and muscle isozymes yielded single bands after polyacrylamide gel electrophoresis in the presence of reducing agents (Fig. 4*A*). In agreement with the isoelectric focusing experiments, the conger muscle enzyme migrated as a more acidic protein than the catfish muscle enzyme (Fig. 4*B*). Pseudoisozymes were observed in the case of both catfish and conger isozymes when reducing agents were not present (Fig. 4, *A* and *B*).

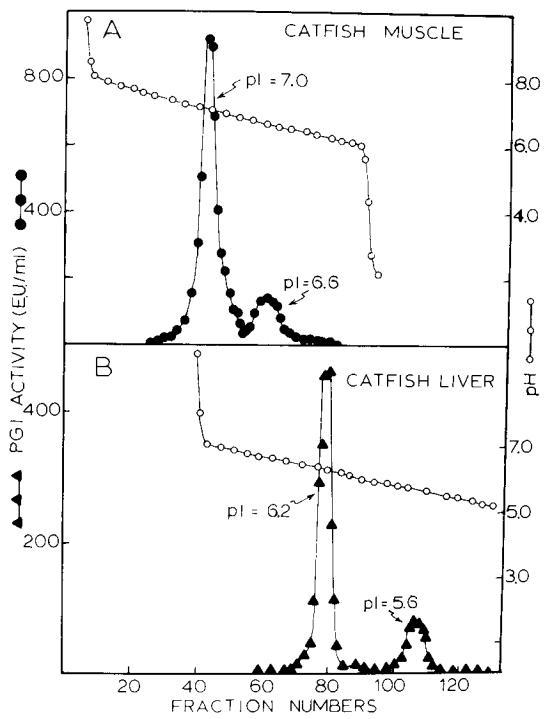

FIG. 3. Isoelectric focusing profiles of catfish muscle and liver glucosephosphate isomerase isozymes. Purified muscle (*A*) and liver (*B*) glucosephosphate isomerases (*PGI*) were subjected to isoelectric focusing at 600 volts and 4° for 72 hours. Ampholines (2% v/v) with pH 6.0 to 8.0 and pH 5.0 to 7.0 were used for the electrophoresis of muscle and liver, respectively. Fractions of 1.0 ml each were collected and assayed for glucosephosphate isomerase activity (● and ▲) and pH (○) as described under "Materials and Methods."

Molecular Weight and Subunit Studies—Although the catfish muscle and liver isozymes exhibited distinctly different electrophoretic, chromatographic, and electrofocusing properties, the two proteins were found to possess essentially identical molecular weights. When the two isozymes were subjected simultaneously to sedimentation velocity ultracentrifugation (Fig. 5), they sedimented as single symmetrical boundaries with identical sedimentation coefficients. The catfish isozymes and conger muscle isozyme yielded the following relationships: catfish muscle $s^0_{20,w}$ = 7.04 (1 − 0.005 C); catfish liver $s^0_{20,w}$ = 7.05 (1 − 0.004 C); conger muscle $s^0_{20,w}$ = 7.00 (1 − 0.004 C), where C is the protein concentration in milligrams per ml. Sedimentation equilibrium ultracentrifugation of the catfish muscle and liver isozymes yielded weight average molecular weights of 132,000 ± 2,000 and 131,000 ± 3,000, respectively.

Polyacrylamide gel electrophoresis of catfish liver and muscle and conger muscle in the presence of sodium dodecyl sulfate yielded single, sharp bands with migrations corresponding to molecular weights of 65,000. Moreover, only a single Coomassie blue-staining band was observed after sodium dodecyl sulfate polyacrylamide electrophoresis of a mixture of equal amounts of human glucosephosphate isomerase and the fish isozymes. These results, as well as the ultracentrifugation studies, suggest that the fish glucosephosphate isomerase isozymes are dimers composed of two identical subunits of M_r = 65,000.

Stability and Catalytic Properties

Stability Studies—The muscle and liver glucosephosphate isomerase isozymes were distinguished on the basis of their stabilities. The purified enzymes were incubated under identical conditions of pH, protein concentration, and ionic strength at various temperatures and immediately placed in ice and assayed for remaining catalytic activity. In addition, the enzymes were incubated at fixed temperatures and the activity monitored as a function of time. In both types of studies the catfish liver isozyme was found to be significantly more stable than the muscle isozyme (Fig. 6). Surprisingly, exactly the reverse situation was found for the conger isozymes. The conger liver isozyme proved to be much more labile than the muscle isozyme. In fact, this particular lability of the conger liver isozyme made isolation and comparative structural studies on the conger liver isozyme difficult.

TABLE I

Isolation of catfish muscle and liver glucosephosphate isomerases

Fractions	Total activity	Total protein	Specific activity	Purification over-all	Recovery over-all
	enzyme units	*mg*	*enzyme units/mg*		%
Catfish muscle (400 g tissue)					
I. Homogenate	41,000	39,900	1.03	(1.0)	(100)
II. 1st DEAE	36,700	2,810	13.1	12.7	89.5
III. 2nd DEAE	35,200	178	198	192	85.8
IV. Isoelectric focusing	21,400	52	412	400	52.2
Catfish liver (100 g tissue)					
I. Homogenate	13,400	11,700	1.15	(1.0)	(100)
II. 1st DEAE	12,300	287	42.7	37.1	91.8
III. 2nd DEAE	10,700	47	226	196	79.9
IV. Sephadex G-200	7,460	18	404	351	55.7
V. Isoelectric focusing	4,650	10	465	404	34.7

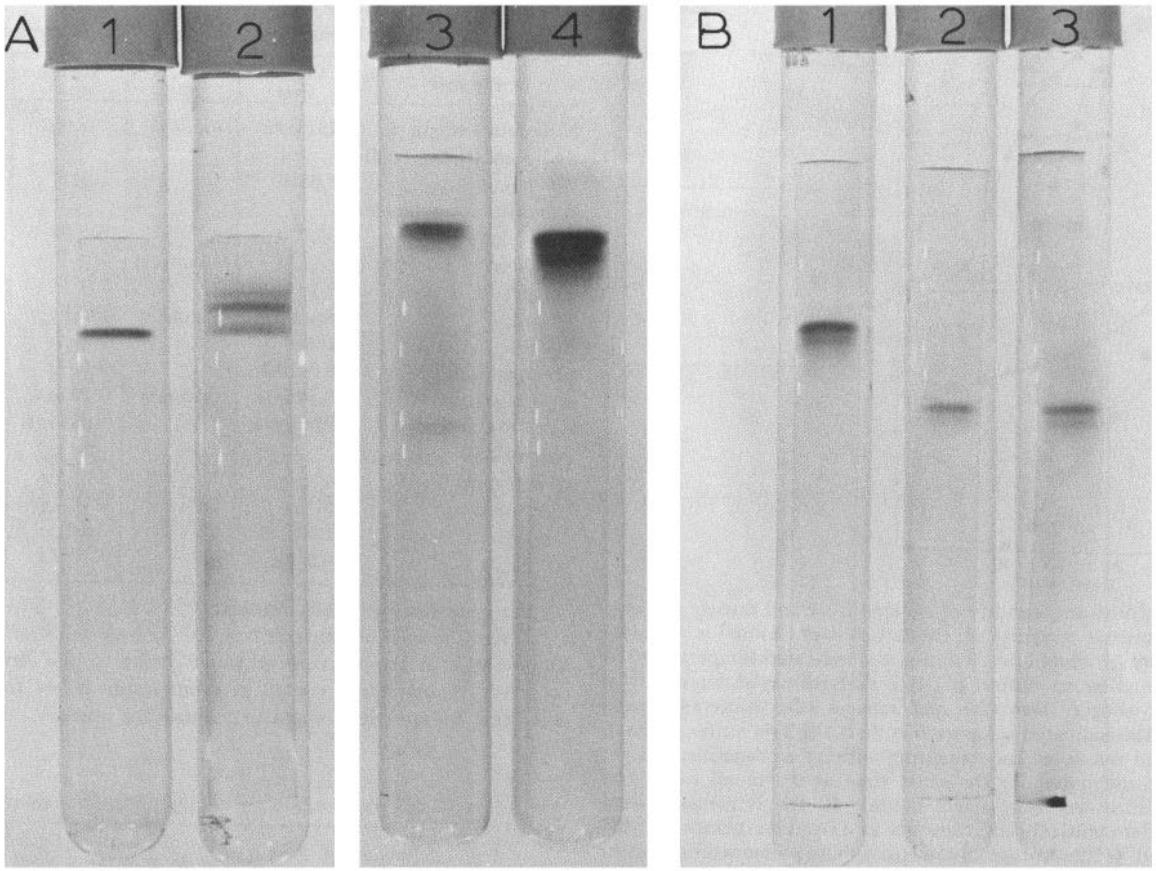

FIG. 4. Polyacrylamide gel electrophoresis of purified glucosephosphate isomerase isozymes. *A*, standard alkaline polyacrylamide gels (7%) were run as described in the text and stained for total protein. The catfish muscle isozyme (specific activity 420 units/mg) applied to the second gel from the left (*2*) had been stored in the absence of reducing agents. After treatment with 5 mM dithiothreitol, the sample was rerun under identical conditions (400 volts, 6 hours) and is shown on the gel on the left (*1*). The catfish liver isozyme (specific activity, 450 enzyme units per mg) after storage in the presence of 5 mM dithiothreitol (*3*) or in the absence of the reducing agent (*4*) was subjected to electrophoresis for 4 hours at 300 volts. All gels were stained for total protein as described under "Materials and Methods." *B*, the isolated isomerases were subjected simultaneously to polyacrylamide gel electrophoresis as above at 400 volts for 8 hours and then stained for total protein as described under "Materials and Methods." *Gel 1* = catfish muscle; *Gel 2* = catfish liver; *Gel 3* = conger muscle.

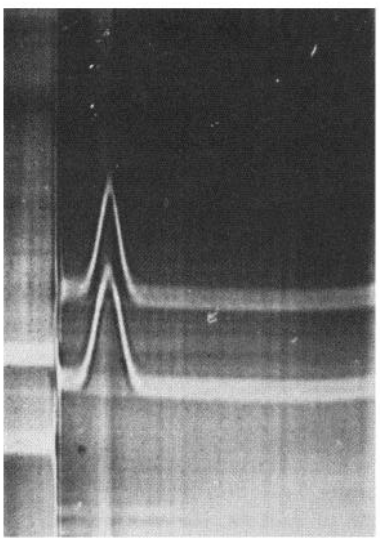

FIG. 5. Sedimentation velocity analytical ultracentrifugation of catfish muscle and liver glucosephosphate isomerases. Catfish liver and muscle isomerases (specific activity, 440 and 460 enzyme units per mg, respectively) were subjected to sedimentation at 60,000 rpm in an AnD rotor. Protein concentrations were 4.0 mg/ml and the buffer was 10 mM triethanolamine (pH 8.0), containing 0.1 M NaCl, 1 mM EDTA, and 0.1% (v/v) 2-mercaptoethanol. Sedimentation was at 18° in standard 12-mm double sector cells with a 1° wedge window employed for one cell. The upper boundary is the muscle isozyme, while the lower is the liver isozyme. The photograph was taken 14 min after reaching speed.

Catalytic Studies—The catfish liver isozyme exhibited a slightly broader pH optimum in the alkaline region than the muscle isozyme (Fig. 7). For example, at pH 10.5, the liver isozyme exhibited 100% maximal activity, whereas the muscle isozyme exhibited only 82% of its maximal activity. Other kinetic parameters of the isozymes were also measured and are summarized in Table II.

Essentially no differences were found in K_m values for fructose 6-phosphate or the competitive inhibitors, 6-phosphogluconate or erythrose 4-phosphate. Activation energies from Arrhenius plots of the isozymes from the two species were nonlinear with breaks occurring at 21–23°. These results are summarized in Table II.

Chemical Properties

Amino Acid Compositions—When the isolated glucosephosphate isomerases from catfish liver and muscle, and from conger muscle, were subjected to amino acid analysis, a high degree of similarity was observed (Table III). The most distinct differences between the catfish liver and muscle isozymes appeared in the contents of lysine, serine, valine, isoleucine,

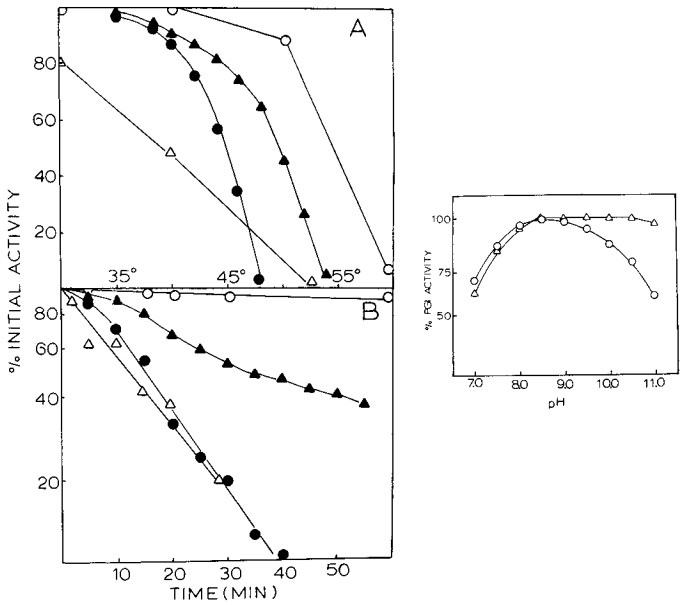

FIG. 6. Temperature stability of isolated liver and muscle glucosephosphate isomerase isozymes. *A*, the catfish liver (▲) and muscle (●) isomerases were incubated for 20 min at the indicated temperatures in 50 mM triethanolamine buffer, pH 8.5, and reassayed for catalytic activity. The conger liver (△) and muscle (○) isomerases were incubated at the indicated temperatures in 0.1 M Tris buffer, pH 8.0, for 10 min and reassayed for remaining activity as described above. Controls were incubated for the same time at 0°. In all cases, the protein concentrations were 0.002 mg/ml. *B*, the isomerases were incubated under identical conditions but at a fixed temperature of 46° for catfish and 44° for conger glucosephosphate isomerase isozymes. Aliquots were removed at the indicated intervals and assayed for remaining activity.

FIG. 7. Effect of pH on the activity of catfish liver and muscle glucosephosphate isomerases. The two isomerases were assayed at 30° in 50 mM triethanolamine buffer and 0.1% (v/v) 2-mercaptoethanol as a function of pH. The pH of each buffer in the cuvette was measured immediately following the assay. Liver and muscle enzyme activities were represented by (△) and (○), respectively.

and phenylalanine. Based on over-all compositions, the catfish liver and muscle enzymes were more similar to each other than either was to the conger muscle isomerase.[1]

Peptide Maps—Tryptic peptide maps of the two catfish isozymes after *S*-carboxymethylation revealed a large number of peptides, far too many to be resolved by this technique. These results were consistent with the high arginine and lysine content of the enzyme (predicted number of peptides = 67 for muscle and 60 for liver).[2] Peptide maps of the catfish muscle and liver isozymes were therefore compared after *S*-carboxymethylation and carbamylation (Fig. 8). In both cases the number of fluorescamine-reacting peptides was in agreement (± two peptides) with the number predicted from the arginine contents. The over-all peptide maps of the two isozymes were very similar and several peptides (*e.g.* Numbers 1, 3, 4, 5, 6, 8, 10, 13, 14) exhibited essentially identical electrophoretic and chromatographic properties. However, distinctly different migrations of the other peptides and the relative fluorescence after reaction with fluorescamine clearly indicated a number of differences in the primary structure of the liver and muscle isozymes. Although a comparison of the peptide maps of the conger (see below) and catfish muscle isozymes suggested some over-all structural homology, a much greater structural homology was evident between the two catfish liver and muscle

[1] It is interesting to note that the ratio of acidic to basic amino acids (as determined from total acid hydrolysates) parallels the isoelectric points of the two catfish isozymes, as well as the enzyme from conger muscle, rabbit, and human. The more acidic properties of the catfish liver isozyme (as determined by isoelectric focusing, electrophoresis, and ion exchange chromatography) are consistent with its higher acidic to basic ratio (1.60) as compared to the muscle isozyme (1.37).

[2] It is of interest to note that both of the catfish and the conger muscle isozymes were digested readily after only *S*-carboxylation, and essentially no "core" material was observed. This is in contrast to tryptic digestion of rabbit (40) or human (9) glucosephosphate isomerases, where complete digestion could only be achieved after both *S*-carboxymethylation and carbamylation.

TABLE II

Comparison of liver and muscle glucosephosphate isomerase isozymes

Property	Catfish liver	Conger liver	Catfish muscle	Conger muscle
Molecular weight	131,000	135,000	132,000	132,000
Sedimentation coefficient ($s^0_{20,w}$)	7.05 S		7.04 S	7.00 S
Subunit molecular weight	65,000		65,000	65,000
Apparent isoelectric pH	6.2		7.0	6.4
Stability[a]	Stable	Labile	Labile	Stable
Specific activity	465		446	422
pH optimum[a]	8.0–10.5		8.0–9.0	
K_m (fructose-6-P)	0.14 mM	0.35 mM	0.20 mM	0.37 mM
K_i (6-P-gluconate)	0.055 mM		0.038 mM	
Activation energy[b] (kcal mol^{-1})				
A	21°	22°	23°	23°
B	12.5	13.4	16.0	17.6
C	6.9	5.3	7.7	5.9

[a] For quantitative details, see text.

[b] Activation energies determined over temperature range of 0–30°. Value for A is temperature at which break in the Arrhenius plot occurred; B, activation energy at temperature below the break; C, activation energies at temperatures above the break.

TABLE III

Amino acid compositions of glucosephosphate isomerases[a]

Amino acid	Catfish muscle	Catfish liver	Conger muscle
Lysine	50.7	42.2	52.6
Histidine	18.1	15.2	13.0
Arginine	15.3	16.5	20.4
Aspartic acid	62.3	65.3	73.2
Threonine	30.8	27.5	43.1
Serine	21.6	30.4	33.7
Glutamic acid	65.2	66.3	61.4
Proline	24.1	29.1	21.2
Glycine	51.3	51.0	58.2
Alanine	45.8	51.6	57.7
Half-cystine[b]	6.2	5.9	N.D.[c]
Valine	52.2	36.3	31.5
Methionine	14.2	14.1	11.7
Isoleucine	31.2	23.0	29.1
Leucine	60.6	65.5	53.2
Tyrosine	11.9	13.4	13.3
Phenylalanine	28.0	35.7	24.7
Tryptophan[d]	11.5	11.8	N.D.

[a] Residues per subunit (65,000).

[b] Determined by reaction with 5,5′-dithiobis (2-nitrobenzoic acid) (35).

[c] N.D. = not determined.

[d] Determined by method of Edelhoch (33).

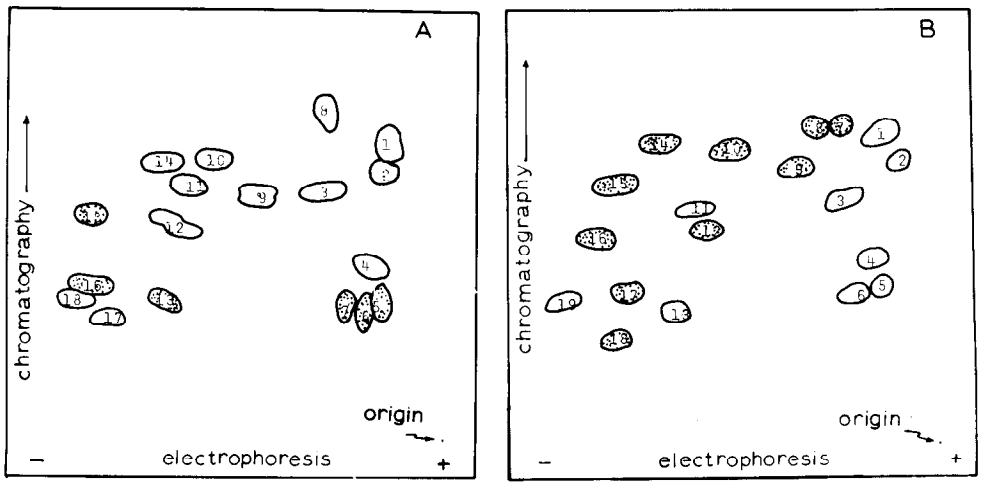

FIG. 8. Tryptic peptide maps of catfish muscle (*A*) and liver (*B*) glucosephosphate isomerase isozymes. After *S*-carboxymethylation and carbamylation, the enzymes were digested with trypsin and peptides mapped on thin layer cellulose as described under "Materials and Methods." The stippled peptides are those which were most highly fluorescent after spraying with fluorescamine.

isozymes than between the two muscle isozymes from the two species.

Glucosephosphate isomerase was isolated from the white muscle of a conger which was heterozygous for the variant allele shown in Fig. 1*B* and compared with the wild type conger muscle enzyme. Isoelectric focusing indicated that the variant muscle allozyme was slightly more basic than the wild type muscle isozyme (*i.e.* apparent pI values of 6.36, 6.42, and 6.55 for the wild type homodimer, the heterodimer, and the variant homodimer, respectively, were obtained). The variant homodimer was collected separately and compared with the wild type conger muscle isozyme by peptide mapping as described above. A composite tryptic peptide map of the wild type and variant homodimers after carboxymethylation and carbamylation is shown in Fig. 9. A single peptide (*e.g.* Number 7 and 7A) was observed with altered electrophoretic mobilities. The altered peptide appears to be more basic and slightly more hydrophobic than the corresponding peptide from the wild type isozyme. These data indicate that the phenotype results from a point mutation.

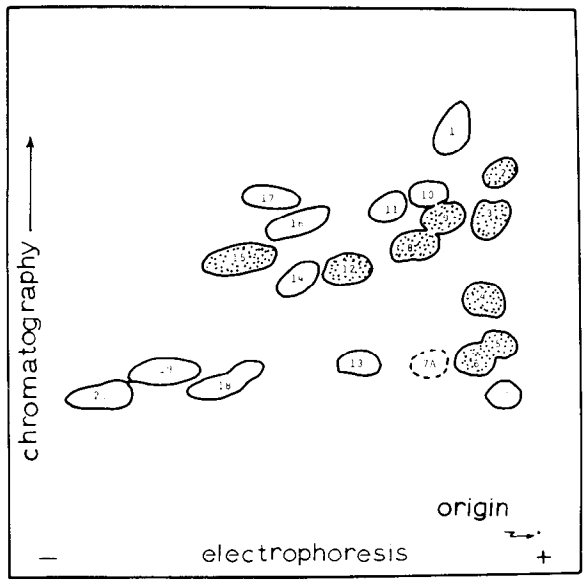

FIG. 9. Composite peptide map, from conger muscle glucosephosphate isomerase and the genetic variant (shown in Fig. 1*B*). The enzymes were *S*-carboxymethylated and α- and ϵ-amino groups carbamylated as described under "Materials and Methods" prior to tryptic digestion. Simultaneous thin layer peptide mapping was carried out, and the plates were sprayed with fluorescamine. The figure represents composite tracings of eight and seven maps of the normal and variant proteins, respectively. The variant protein was identical with the normal protein with the exception of Peptide 7 which was present in the wild type and was replaced by Peptide 7A in the genetic variant. The most highly fluorescent peptides are shown by stippling.

DISCUSSION

In recent years studies on glucosephosphate isomerase have centered on structural and kinetic properties of the mammalian and yeast enzymes. Genetic studies on glucosephosphate isomerase in a number of mammalian species (3, 8, 11) are consistent with the hypothesis that the enzyme structure is determined by one or more alleles at a single autosomal locus. It was, therefore, of great interest to find that in diverse teleostean species there are two gene loci for glucosephosphate isomerase which result in isozymes with marked tissue specificity.

In both the conger and catfish most tissues synthesize the isozyme referred to in this study as the "liver-type," while white muscle was found to possess almost exclusively the more basic "muscle-type" isomerase. The small amounts of liver-type isozyme observed in white muscle may be the result of contamination by serum or lymph. Red muscle and serum appear to contain both types of isozymes, as well as hybrids, suggesting that both types of polypeptides may be synthesized in some cells.

The distinct chemical and physical properties determined for the isolated muscle and liver isozymes clearly show that the two forms of glucosephosphate isomerase are indeed of genetic origin and do not represent post-transcriptional modifications such as proteolysis, deamidation, or covalent modification. These data corroborate the genetic studies carried out on tissue extracts from these and other species of teleosts. On the other hand, artifactual multiple electrophoretic forms of fish glucosephosphate isomerase occur if caution is not taken to maintain the enzyme in a reduced state. This situation closely parallels the "pseudoisozymes" observed with glucosephosphate isomerase from rabbit muscle (15), human and rat (16). The faint, rapidly migrating anodal bands of glucosephosphate isomerase activity observed upon starch gel electrophoresis of extracts (Fig. 1) may be such an example.

The specific activities of the isolated glucosephosphate isomerase isozymes from the fish were approximately 50% lower than those found for the enzyme isolated from human or rabbit muscle (9, 15) or yeast (41). This does not, however,

appear to be the result of heterogeneity, since both liver and muscle isozymes were shown to be homogeneous by a variety of criteria. Likewise, the specific activities of 400 to 500 units/mg do not seem to be the result of partial denaturation during the isolation processes. The two isolation procedures were developed totally independently in our two laboratories with comparable results. While it might be argued that the heat step involved in the purification of the conger glucosephosphate isomerase may have induced some partial denaturation or proteolysis, this cannot be the case for the catfish isozymes. The procedure outlined for the isolation of the catfish isozymes is mild, and recoveries were good at all stages.[3] It thus appears that the glucosephosphate isomerases from teleosts do, indeed, possess lower specific activities than the enzyme from the higher mammals thus far studied. The difference in the stability of the muscle and liver isozymes was most striking in the conger where the liver isozyme was much more labile than the muscle isozyme. Crude homogenates of cod similarly have shown a more labile liver isozyme, and Avise and Kitto (23) reported a more labile liver glucosephosphate isozyme in extracts of *Astynax mexicanus*. It is not understood why the isolated liver isozyme of the catfish does not show this lability. It is possible that the inactivation of the liver isozyme in crude extracts or partially purified fractions may be due to protease contamination.

The amino acid compositions and peptide maps clearly indicated that a number of structural differences in the two types of isozymes exist. The catfish liver and muscle isozymes were more similar to each other (amino acid composition coefficient (43) of 0.95) than either isozyme was to the conger muscle protein (composition coefficient of catfish muscle *versus* conger muscle = 0.90; and catfish liver *versus* conger muscle = 0.92). This was corroborated further by comparison of the tryptic peptide maps from the isozymes of the two species. The conger muscle glucosephosphate isomerase was essentially as different in its amino acid composition from the two catfish enzymes as it was from rabbit enzyme (cc = 0.92) or human enzymes (cc = 0.91). This is perhaps not surprising in view of the probable separation of the Elopomorpha from the Euteleostei as early as the late Jurassic (44). However, the true evolutionary relationships between the enzymes must await sequence studies.

The observation of a single peptide difference in the conger muscle glucosephosphate isomerase variant parallels the recent findings with a human glucosephosphate isomerase variant (9) and is consistent with a point mutation affecting a single amino acid residue. Previous studies (25) indicate widespread allelic variation at both glucosephosphate isomerase loci in many teleosts. The mutation studied in this case resulted in an increase of approximately 0.2 in the apparent isoelectric point. Although it is difficult to correlate pI changes directly with charge changes in the protein, such a change of 0.2 is more consistent with a neutral → basic or acidic → neutral amino acid transition than an acidic → basic substitution (9).

Glucosephosphate isomerase has long been considered a bifunctional enzyme of glycolysis and gluconeogenesis and present in large excess compared to the rates of metabolic flux. Thus, in most organisms studied (19) the necessity of tissue-specific isozymes has not evolved. The teleosts appear to be an exception with tissue-specific, genetically determined isozymes. Avise and Kitto (23) have suggested that the high degree of polyploidy in teleosts is a likely cause of gene multiplicity of glucosephosphate isomerase.[4]

The present study has shown that indeed true isozymes exist in catfish and conger, and that these isozymes exhibit a tissue-specific distribution. The muscle- and liver-type enzymes clearly have experienced a number of amino acid sequence changes during the course of evolution since gene duplication. Although the unique physiological functions of each isozyme remain to be elucidated, it is clear that further studies on the two isozymes and the apparent widespread allelic variants of these genes should provide a unique opportunity to assess the metabolic roles of the enzyme in various tissues and to correlate structure-function relationships.

REFERENCES

1. Carter, N., and Dando, P. (1973) *Biochem. Soc. Trans.* **1,** 1263–1264
2. Carter, N. D., and Parr, C. W. (1967) *Nature* **216,** 511
3. DeLorenzo, R. J., and Ruddle, F. H. (1959) *Biochem. Genet.* **3,** 151–162
4. Welch, S. G., Fitch, L. I., and Parr, C. W. (1970) *Biochem. J.* **117,** 525–531
5. Saison, R. (1968) *Proceedings of the Eleventh European Conference on Animal Blood Groups and Biochemical Polymorphisms*, pp. 321–330
6. Andresen, A. (1970) *Anim. Blood Grps. Biochem. Genet.* **1,** 173–174
7. Detter, J. C., Ways, P. O., Giblett, E. R., Baughn, M. A., Hopkinsons, D. A., Povy, S., and Harris, H. (1968) *Ann. Human Genet.* **31,** 329–338
8. Welch, S. G. (1971) *Human Hered.* **21,** 467–477
9. Tilley, B. E., Gracy, R. W., and Welch, S. G. (1974) *J. Biol. Chem.* **249,** 4571–4579
10. Carter, N. D., Hill, M. R., and Weir, B. J. (1972) *Biochem. Genet.* **6,** 147–156
11. Baich, A., Wolfe, R. G., and Reithel, F. J. (1960) *J. Biol. Chem.* **235,** 3130–3133
12. Mathers, N. F., Wilkins, N. P., and Walne, P. R. (1974) *Biochemical Systematics and Ecology* **2,** 93–96
13. Kempe, T. D., Gee, D. M., Hathaway, G. M. and Noltman, E. A. (1974) *J. Biol. Chem.* **249,** 4625–4633
14. Selander, R. K., Yang, S. R., Lewontin, R. C., and Johnson, W. E. (1970) *Evolution* **24,** 402
15. Blackburn, M. N., Chirgwin, J. M., James, G. T., Kempe, T. D., Parsons, T. F., Register, A. M., Schnackerz, K. D., and Noltmann, E. A. (1972) *J. Biol. Chem.* **247,** 1170–1179
16. Payne, D. M., Porter, D. W., and Gracy, R. W. (1972) *Arch. Biochem. Biophys.* **151,** 122–127
17. Sasaki, T., Hosoda, H., Tadokoro, K., and Suzuki, S. (1972) *Phytochemistry* **11,** 2981–2983
18. Sasaki, T., Tadokoro, K., and Suzuki, S. (1972) *Biochem. J.* **129,** 789–791
19. Gracy, R. W. (1974) *Proceedings of the Third International Conference on Isozymes*, vol. 1, pp. 471–487, Academic Press, New York
20. Avise, J. C., and Selander, R. K. (1972) *Evolution* **26,** 1–19
21. Yndgaard, C. F. (1972) *Hereditas* **71,** 151–154
22. Wilkins, N. P. (1972) *J. Fish. Biol.* **4,** 487–504
23. Avise, J. C., and Kitto, G. B. (1973) *Biochem. Genet.* **8,** 113–132
24. Johnson, A. G., Utter, F. M., and Hodgins, H. O. (1973) *Comp. Biochem. Physiol.* **44B,** 397–406
25. Dando, P. R. (1974) *Comp. Biochem. Physiol.* **47B,** 663–679
26. Schnidtke, J., and Engel, W. (1974) *Humangenetik* **21,** 39–45
27. Gracy, R. W., and Tilley, B. E. (1974) *Methods Enzymol.* **41B,**

[3] In fact, the isolation procedure described here for the catfish isozymes is milder than the procedure conventionally employed for the isolation of the rabbit muscle enzyme (42).

[4] The two autosomal loci for glucosephosphate isomerase in place (*Pleuronectes platessa* L.) do not appear to be linked (P. R. Dando and C. E. Purdom, unpublished experiments).

392–399
28. Wilkinson, G. N. (1961) *Biochem. J.* **80,** 324–332
29. Cleland, W. W. (1967) *Adv. Enzymol.* **29,** 1–32
30. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **177,** 751–766
31. Moore, S., and Stein, W. H. (1963) *Methods Enzymol.* **6,** 821–831
32. Spackman, D. H., Moore, S., and Stein, W. H. (1958) *Anal. Chem.* **30,** 1190 1206
33. Edelhoch, H. (1967) *Biochemistry* **6,** 1948–1954
34. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77
35. Yphantis, D. A. (1964) *Biochemistry* **3,** 297–317
36. Scopes, R. K. (1968) *Biochem. J.* **107,** 139–150
37. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404–427
38. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412
39. Sawyer, T. H., Tilley, B. E., and Gracy, R. W. (1972) *J. Biol. Chem.* **247,** 6499–6505
40. James, G. T., and Noltmann, E. A. (1973) *J. Biol. Chem.* **248,** 730–737
41. Nakagawa, Y., and Noltmann, E. A. (1967) *J. Biol. Chem.* **242,** 4782–4788
42. Noltmann, E. A. (1966) *Methods Enzymol.* **9,** 557–568
43. Dedman, J. R., Gracy, R. W., and Harris, B. G. (1974) *Comp. Biochem. Physiol.* **49B,** 715–731
44. Florey, P. L. (1973) *Zool. J. Linn. Soc.* **53,** Suppl. 1, 351–368