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Phosphoglucose Isomerase Gene Duplication in the Bony Fishes: An Evolutionary History

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Electrophoretic patterns of phosphoglucose isomerase (PGI) in bony fishes provide strong evidence for a model of genetic control by two independent structural gene loci, most likely resulting from a gene duplication. This model is confirmed by a comparison of certain kinetic and molecular properties of the PGI homodimers (PGI-1 and PGI-2) isolated from extracts of the teleost Astyanax mexicanus. *In addition, in most higher teleosts examined, the PGI enzymes show a regular pattern of tissue distribution, with PGI-2 predominant in muscle, the heterodimer often strongest in the heart, and PGI-1 predominant in liver and other organs. An examination of 53 species of bony fishes belonging to 38 families indicates a widespread occurrence of duplicate PGI loci and an early origin of the gene duplication, perhaps in the Leptolepiformes. The apparent presence of three PGI loci in trout and goldfish exemplifies how new loci can be incorporated into the genome through polyploidization.*

INTRODUCTION

Almost all vertebrates and invertebrates thus far studied possess only a single phosphoglucose isomerase (PGI) gene locus: man (Detter *et al.*, 1968), deer (Ramsey *et al.*, 1972), rodents (Carter and Parr, 1967; Selander

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et al., 1969; DeLorenzo and Ruddle, 1969; Johnson and Selander, 1971; Johnson *et al.*, 1972), birds (Nottebohm and Selander, 1972), lizards (Webster *et al.*, 1972), frogs (Ralin *et al.*, 1972), horseshoe crabs (Selander *et al.*, 1970), fiddler crabs (Selander *et al.*, 1972), and certain insects (Avise and Selander, 1972; Ramsey and Avise, unpublished).

Yoshida and Carter (1969) observed multiple electrophoretic bands of phosphoglucose isomerase, representing at least two structurally different isozymes, in rabbit hemolysates and muscle extracts. However, the number of genetic loci controlling PGI was not determined since all specimens showed identical patterns on the gels. Similarly, the genetic basis of three isozymes observed by Nakagawa and Noltmann (1967) in brewers and bakers yeast was not determined.

Avise and Selander (1972) describe allozymic patterns of phosphoglucose isomerase in a characid fish (*Astyanax mexicanus*) that are consistent with a model of control by two independent structural gene loci, most likely resulting from a gene duplication. In this paper, we present the evidence on which this conclusion is based, including an analysis of certain kinetic and molecular properties of the PGI homodimers in *A. mexicanus*. On the basis of a study of the tissue distribution and occurrence of multiple PGI loci in a wide variety of bony fishes (Osteichthyes), we propose a hypothesis of the time of origin of the gene duplication and the course of the divergent specialization of the homodimeric and heterodimeric enzyme forms.

MATERIALS AND METHODS

Specimens of *A. mexicanus* used in the extraction of PGI for characterization were collected in Cueva del Pachon, near Ciudad Mante, Tamaulipas, Mexico (see Avise and Selander, 1972). Other species were collected in Texas and Massachusetts or purchased from commercial dealers. All fish were stored on dry ice immediately after capture.

Fructose 6-phosphate (F6P), glucose 6-phosphate dehydrogenase (G6PDH), triphosphopyridine nucleotide (NADP), phenazine methosulfate (PMS), MTT tetrazolium (MTT), 6-phosphogluconic acid, and EDTA disodium salt were obtained from Sigma Chemical Company, phosphoenol pyruvic acid from Calbiochem, adenosine 5′-triphosphate from Pabst Laboratories Biochemical, and Electrostarch lot 171 from Otto Hiller, Madison, Wisconsin. Other chemicals were of reagent grade.

Starch Gel Electrophoresis

Extracts for electrophoresis were prepared by homogenizing the whole animal or tissue in a glass tissue grinder with an equivalent volume of buffer

(0.01 M tris, 0.01 M EDTA, 5×10^{-5} M NADP, *p*H adjusted to 6.8 with HCl). The homogenate was centrifuged at $49{,}000 \times g$ for 40 min, and the supernatant was stored at -60 C.

Horizontal starch gel electrophoresis was carried out as described by Selander *et al.* (1971, appendix), using either a Poulik (*p*H 8.7)–borate (*p*H 8.2) discontinuous buffer system at 25 v/cm for $2\frac{1}{2}$ hr, or a potassium phosphate (*p*H 6.7) continuous buffer at 13 v/cm for 6 hr. The enzyme was localized with a staining mixture described by Selander *et al.* (1971). All PGI bands appear anodal to the origin.

Isolation of the Homodimers

Isolation of the *Astyanax* PGI homodimers was carried out using an electrophoretic technique similar to that described by Prasad and Wright (1971). After electrophoresis, one slice of the gel was stained for PGI activity. The stained slice was then superimposed on the corresponding remainder of the gel, and a cut was made through the gel just anodal to each homodimer, using the stained slice as a guide. A small sponge was placed in the slit, and a strip of dialysis tubing was inserted between the sponge and the gel, just anodal to the sponge. The gel was then electrophoresed for $\frac{1}{2}$ hr, allowing each homodimer to run up into its appropriate sponge (the dialysis tubing prevented the enzyme from leaving the sponge). The enzyme was recovered by washing the sponge in a small volume of buffer.

Enzyme Assay

Enzymatic activity was measured at room temperature by the spectrophotometric assay procedure of Noltmann (1964). A Zeiss PMQ II spectrophotometer was used to follow the change in absorbance at 340 mμ caused by NADPH formation in a coupled enzyme system (F6P substrate and G6PDH indicator enzyme). The assay solution before addition of phosphoglucose isomerase enzyme solution contained 0.138 M tris, 0.023 M $MgCl_2$, 4×10^{-4} M F6P, 7.4×10^{-5} M NADP, and 1.96 units G6PDH. Addition of 0.1 ml PGI solution brought the final assay volume to 3 ml.

The activity of each of the two forms of PGI was measured over varying concentrations of inhibitor. The inhibitor and PGI were allowed to mix for 1 min before being added to the remainder of the assay solution. Michaelis constants for PGI homodimers were determined by varying the concentration of F6P.

Heat Inactivation

Thermal stabilities of the PGI homodimers were studied by heating the enzymes (a) at different temperatures for 5 min and (b) at 45 C for different

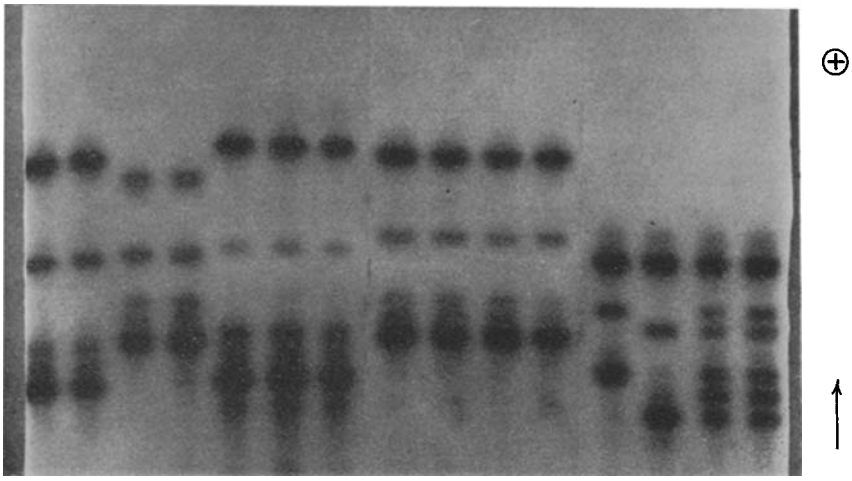

Fig. 1. Phenotypes of phosphoglucose isomerases from whole-animal extracts. Left to right: Two homozygous bluegill, two homozygous orangespotted sunfish, three homozygous silversides, four homozygous largemouth bass, and four mosquitofish with presumed phenotypes as follows: *Pgi-1*^{a}/*Pgi-1*^{a}, *Pgi-2*^{a}/*Pgi-2*^{a}; *Pgi-1*^{a}/*Pgi-1*^{a}, *Pgi-2*^{b}/*Pgi-2*^{b}; *Pgi-1*^{a}/*Pgi-1*^{a}, *Pgi-2*^{a}/*Pgi-2*^{b}; and *Pgi-1*^{a}/*Pgi-1*^{a}, *Pgi-2*^{a}/*Pgi-2*^{b}.

periods. After heating, the enzyme solutions were chilled on ice, and remaining PGI activity was assayed at room temperature.

RESULTS

Electrophoretic Analysis

In fish tissues, the patterns of PGI activity on the gels are similar to those for the multiple loci encoding malate dehydrogenase in several fishes (Bailey *et al.*, 1970; Whitt, 1970; Avise and Selander, 1972). Typically, individuals homozygous at both PGI loci show three widely spaced bands (see Fig. 1). (Other paler bands slightly anodal to the slowest are interpreted as secondary or satellite bands.) Presumably, the three bands result from association of two types of polypeptide subunits, one type encoded by each locus, to produce AA, AB, and BB dimers. The faster-migrating, more anodal band represents the homodimer produced by association of A subunits encoded by locus *Pgi-1*, and the slower band represents the homodimer produced by association of B subunits encoded by locus *Pgi-2*. (In the following discussion, PGI-1 and PGI-2 will specifically refer to the homodimeric PGI enzymes encoded by *Pgi-1* and *Pgi-2*, respectively.) Since the intermediate heterodimer band stains less intensely than those representing the homodimers, the association

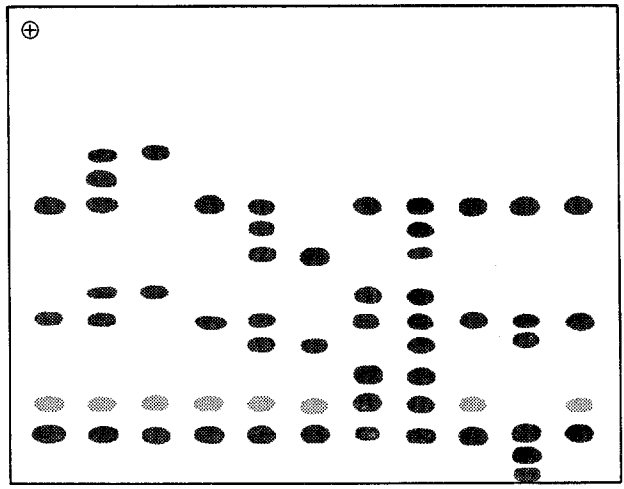

Fig. 2. Diagrammatic representation of some of the phenotypes observed in whole-animal extracts of *A. mexicanus*. Presumed phenotypes from left to right: *Pgi-1*^{b}/*Pgi-1*^{b}, *Pgi-2*^{b}/*Pgi-2b*; *Pgi-1*^{a}/*Pgi-1*^{b}, *Pgi-2*^{b}/*Pgi-2*^{b}; *Pgi-1*^{a}/*Pgi-1*^{a}, *Pgi-2*^{b}/*Pgi-2*^{b}; *Pgi-1*^{b}/*Pgi-1*^{b}, *Pgi-2*^{b}/*Pgi-2*^{b}; *Pgi-1*^{b}/*Pgi-1*^{c}, *Pgi-2*^{b}/*Pgi-2*^{b}; *Pgi-1*^{c}/*Pgi-1*^{c}, *Pgi-2*^{b}/*Pgi-2*^{b}; *Pgi-1*^{b}/*Pgi-1*^{b}, *Pgi-2*^{a}/*Pgi-2*^{b}; *Pgi-1*^{b}/*Pgi-1*^{c}, *Pgi-2*^{a}/*Pgi-2*^{b}; *Pgi-1*^{b}/*Pgi-1*^{b}, *Pgi-2*^{b}/*Pgi-2*^{b}; *Pgi-1*^{b}/*Pgi-1*^{b}, *Pgi-2*^{b}/*Pgi-2*^{c}; and *Pgi-1*^{b}/*Pgi-1*^{b}, *Pgi-2*^{b}/*Pgi-2*^{b}.

of subunits in the whole organism is probably not random. Alternatively, the heterodimer may be less stable *in vivo*.

Individuals heterozygous at the PGI loci exhibit phenotypic patterns fully consistent with the above model. Fish heterozygous at a single locus show six bands, representing all possible associations of three types of subunits as follows: AA, A′A, A′A′, AB, A′B, and BB (see Figs. 1 and 2). Individuals heterozygous at both loci often show only nine bands, rather than the ten predicted by random association of four types of subunits, but most likely this is due to overlap in mobility of some of the isozymes.

Tissue Distribution

Patterns of tissue and organ distribution of the PGI enzymes were examined in 16 species belonging to 15 families of Osteichthyes (Table I). Blood, eye, eggs, brain, liver, kidney, heart, gonad, spleen, skeletal muscle, gills, intestine, gallbladder, and pyloric cecae were all examined in a few of the species, although muscle, heart, and liver proved to be most informative. The organs and tissues were homogenized separately and electrophoresed as described above. A typical pattern of tissue distribution of PGI is shown in Fig. 3.

Table I. Species of Fish Examined for Phosphoglucose Isomerase

Superorder Order	Family	Scientific name	Common name	Sample size	Probable No. loci	Individual tissues examined	Heterozygotes observed
Semionotiformes	Lepisosteidae	*Lepisosteus oculatus*	Spotted gar	1	1	Yes	No
		Lepisosteus platostomus	Shortnose gar	2	1	Yes	Yes
Amiiformes	Amiidae	*Amia calva*	Bowfin	1	1	Yes	No
Elopomorpha							
Anguilliformes	Congridae	*Congrina flava*	Yellow conger	1	2	Yes	No
Clupeomorpha							
Clupeiformes	Clupeidae	*Brevoortia tyrannus*	Atlantic menhaden	2	$\geqslant 1$	No	Yes
		Clupea harengus	Atlantic herring	2	$\geqslant 1$	No	Yes
		Dorosoma cepedianum	Gizzard shad	6	$\geqslant 1$	Yes	No
Protacanthopterygii							
Salmoniformes	Salmonidae	*Salmo gairdneri*	Rainbow trout	30	3	Yes	No
		Salmo trutta	Brown trout	5	3	Yes	No
		Salvelinus fontinalis	Brook trout	5	3	No	No
	Esocidae	*Esox americanus*	Grass pickerel	2	2	No	No
Myctophiformes	Synodontidae	*Synodus foetens*	Inshore lizardfish	1	$\geqslant 1$	No	No
Ostariophysi							
Cypriniformes	Characidae	*Astyanax mexicanus*	Mexican tetra	400	2	Yes	Yes
	Cyprinidae	*Notropis venustus*	Blacktail shiner	2	2	No	Yes
		Carassius auratus	Goldfish	6	3	Yes	Yes
Siluriformes	Ictaluridae	*Ictalurus melas*	Black bullhead	1	2	No	No
	Ariidae	*Arius felis*	Sea catfish	2	2	Yes	Yes
Paracanthopterygii							
Batrachoidiformes	Batrachoididae	*Porichthys porosissimus*	Atlantic misdhipman	1	$\geqslant 1$	No	No
Gadiformes	Gadidae	*Microgadus tomcod*	Atlantic tomcod	1	2	Yes	Yes
	Ophidiidae	*Rissola marginata*	Striped cusk-eel	1	2	No	No
	Zoarcidae		Eelpout	1	2	Yes	No

Atherinomorpha	Cyprinodontidae	*Cyprinodon variegatus*	Sheepshead minnow	2	2	No	No
		Fundulus heteroclitus	Mummichog	1	2	No	No
	Poeciliidae	*Gambusia geiseri*	Largespring gambusia	15	2	No	No
		Gambusia affinis	Mosquitofish	15	2	No	Yes
		Gambusia heterochir	Clear Creek gambusia	1	2	No	Yes
		Poecilia formosa	Amazon molly	3	2	No	No
	Atherinidae	*Menidia menidia*	Atlantic silverside	2	2	No	Yes
Acanthopterygii							
Gasterosteiformes	Syngnathidae	*Syngnathus fuscus*	Northern pipefish	1	1	No	No
Perciformes	Serranidae	*Centropristis striata*	Black sea bass	1	2	Yes	No
	Centrarchidae	*Lepomis cyanellus*	Green sunfish	30	2	No	Yes
		Leptomis macrochirus	Bluegill	2000	2	No	No
		Lepomis humilis	Orangespotted sunfish	40	2	No	Yes
		Micropterus salmoides	Largemouth bass	5	2	No	No
	Percidae	*Percina caprodes*	Logperch	1	2	No	No
	Pomatomidae	*Pomatomus saltatrix*	Bluefish	1	$\geqslant 1$	No	No
	Pomadasyidae	*Orthopristis chrysoptera*	Pigfish	1	2	Yes	No
	Sparidae	*Stenotomus chrysops*	Scup	1	2	Yes	No
	Sciaenidae	*Cyoscion nothus*	Silver seatrout	1	2	No	No
		Micropogon undulatus	Atlantic croaker	1	2	No	No
	Cichlidae	*Cichlasoma cyanoguttatum*	Rio Grande perch	1	2	No	No
	Labridae	*Tautoga onitis*	Tautog	1	2	Yes	No
		Tautogolabrus adspersus	Cunner	2	2	No	No
	Mugilidae	*Mugil cephalus*	Striped mullet	4	2	Yes	No
	Trichiuridae	*Trichiurus lepturus*	Atlantic cutlassfish	1	2	No	No
	Scombridae	*Scomber scombrus*	Atlantic mackerel	2	2	No	No
	Stromateidae	*Peprilus triacanthus*	Butterfish	1	2	No	No
	Triglidae	*Prionotus* sp.	Sea Robin	2	2	No	No
	Cottidae	*Hemitripterus americanus*	Sea raven	2	2	Yes	No
Pleuronectiformes	Bothidae	*Ancylopsetta quadrocellata*	Ocellated flounder	1	2	No	No
	Soleidae	*Achirus lineatus*	Lined sole	1	2	No	Yes
	Cynoglossidae	*Symphurus plagiusa*	Blackcheek tonguefish	1	$\geqslant 1$	No	No
Tetraodontiformes	Tetraodontidae	*Sphoeroides parvus*	Least puffer	1	2	No	Yes

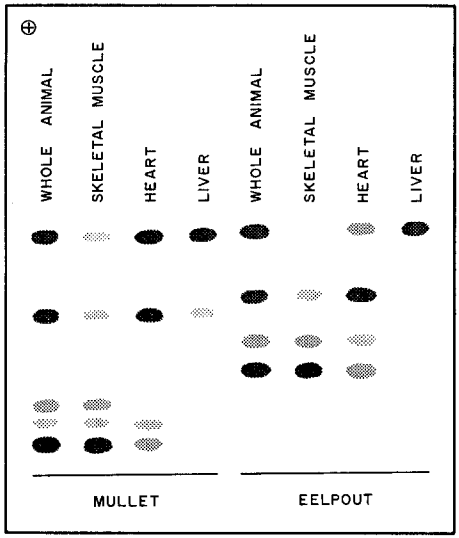

Fig. 3. Diagrammatic representation of tissue distribution of phosphoglucose isomerases in typical teleosts.

In all species possessing two PGI loci (with the exception of the yellow conger, *Congrina flava*), the slower-migrating PGI-2 homodimer is strongly expressed in skeletal muscle and is weak or absent in other tissues. PGI-1 is very weak in muscle. The heterodimer is strongly expressed in the heart, often along with moderate PGI-1 and PGI-2 activity. All other tissues and organs predominantly exhibit PGI-1. In the yellow conger, *C. flava*, white muscle and eggs express both homodimer bands, while heart and liver show mainly PGI-1.

Characterization of PGI in *A. mexicanus*

In order to substantiate more fully kinetic and molecular differences of the PGI enzymes and hence their genetic control by independent gene loci, we chose to examine in more detail the properties of the PGI homodimers in the characid teleost *A. mexicanus*. Both PGI loci are polymorphic in *A. mexicanus*. Populations along a 50 mile transect in northeastern Mexico exhibit eight alleles at the *Pgi-1* locus and six alleles at the *Pgi-2* locus. The high frequency of heterozygotes, the close correspondence of genotypes at both loci with Hardy–Weinberg expectations, and the occurrence of almost all possible combinations of alleles at the two loci in the populations sampled provide additional evidence for the presence and independence of two genetic loci.

The homodimeric forms of PGI were isolated from specimens of *A. mexicanus* as described above. Heat inactivation experiments demonstrate

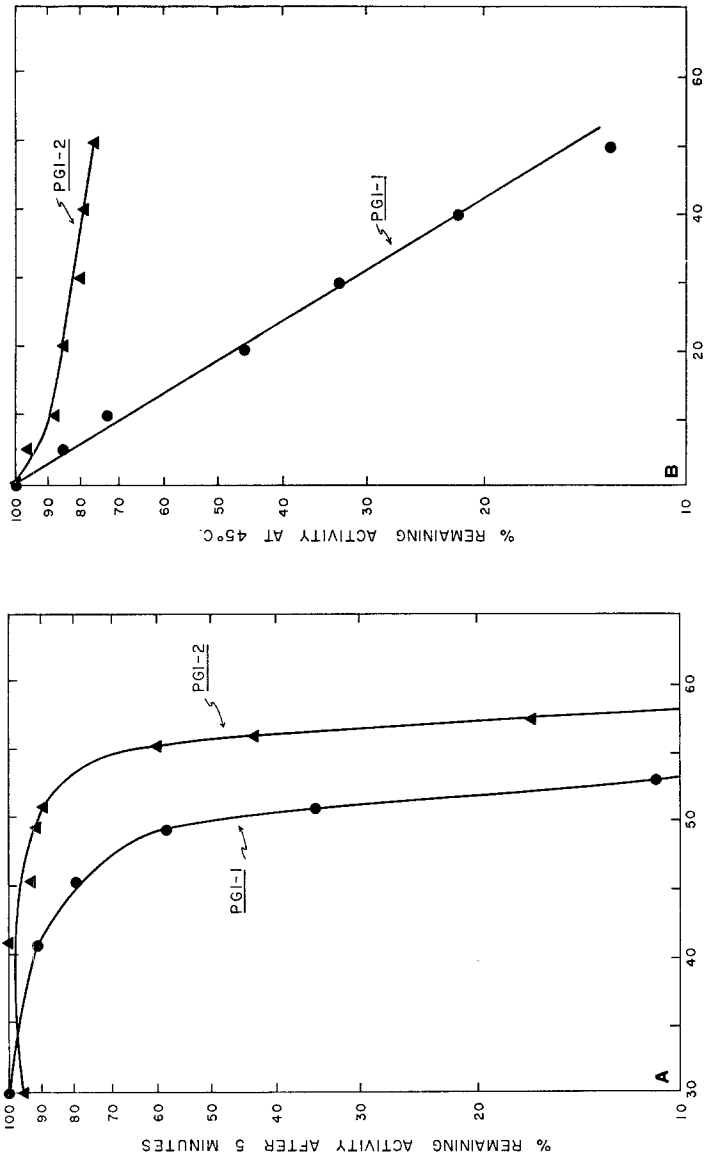

Fig. 4. Relative heat stabilities of phosphoglucose isomerase homodimers from *A. mexicanus*. A: Heating at different temperatures for 5 min. B: Heating at 45 C for different periods.

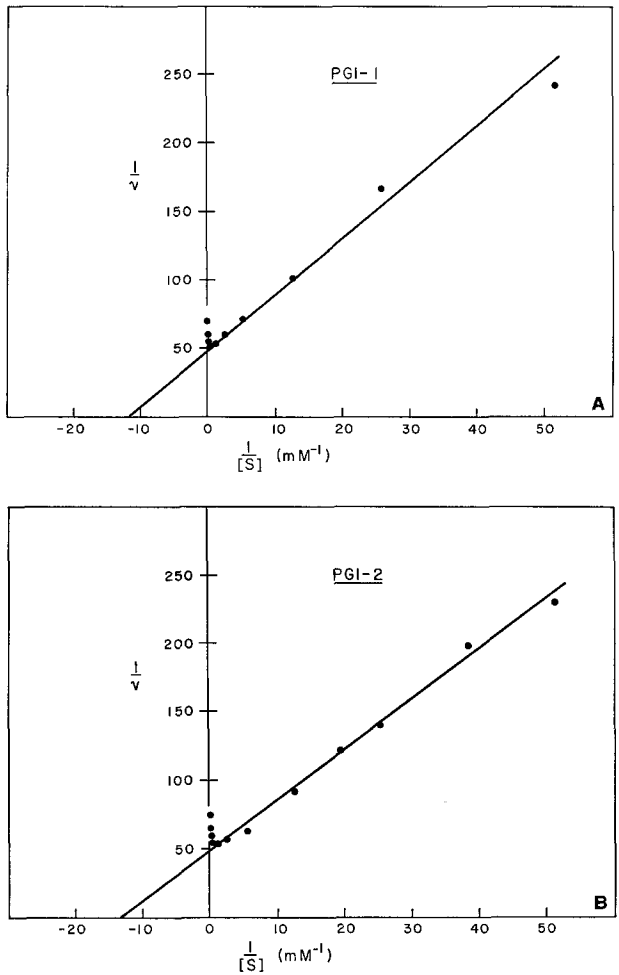

Fig. 5. Double reciprocal plots of *A. mexicanus* phosphoglucose isomerase homodimers with fructose 6-phosphate as varied substrate. A: PGI-1. B: PGI-2.

striking differences in the thermal stabilities of the two forms (Fig. 4). PGI-1 and PGI-2 lose 90% and 23% of their activity, respectively, after being heated at 45 C for 50 min. Furthermore, all PGI-1 activity is lost after 5 min of heating at 53 C, whereas 85% of PGI-2 activity remains.

Michaelis constants for the PGI homodimers were determined from Lineweaver–Burk reciprocal plots, using noninhibitory ranges of F6P concentration (Fig. 5). The K_m values for F6P are almost identical in PGI-1 and PGI-2: 7.0×10^{-5} and 7.6×10^{-5}, respectively. These values are com-

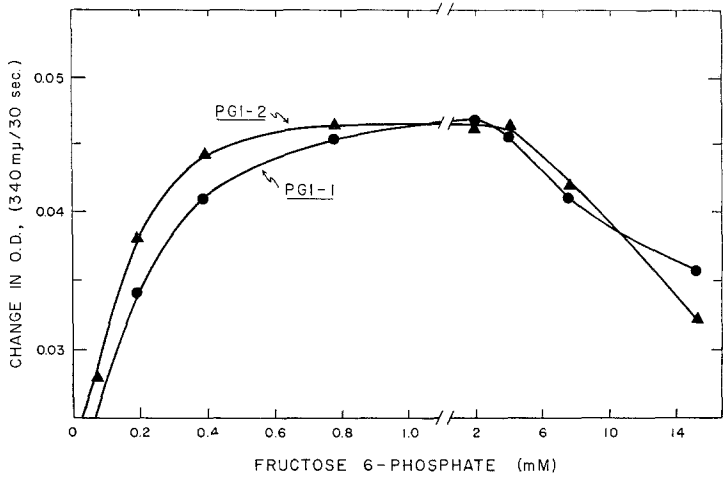

Fig. 6. Substrate inhibition of *A. mexicanus* phosphoglucose isomerase homodimers by fructose 6-phosphate.

parable to those found for the enzymes from bovine mammary gland (Hines and Wolfe, 1963), rabbit brain and skeletal muscle, and human erythrocytes (Kahana *et al.*, 1960). Both PGI-1 and PGI-2 are inhibited by high F6P substrate concentrations (Fig. 6).

The action of isomerase inhibitors on the activity of PGI homodimers from *Astyanax* extracts is shown in Table II. Phosphoenol pyruvate (PEP) and adenosine 5′-triphosphate (ATP) inhibit both PGI forms, but 6-phosphogluconate apparently has little effect at concentration 1×10^{-4} M. The inhibitory effect of higher concentrations of ATP appears to be greater on PGI-1 than on PGI-2.

Table II. Effect of Inhibitors on Phosphoglucose Isomerase Homodimers Isolated from *A. mexicanus*

Inhibitor	Concentration (*M*)	Percent Inhibition of PGI-1	Percent Inhibition of PGI-2
Phosphoenol pyruvate	1×10^{-3}	8	21
	2.5×10^{-3}	46	46
	5×10^{-3}	90	98
Adenosine 5′-triphosphate	1×10^{-3}	3	10
	2.5×10^{-3}	13	22
	4×10^{-3}	32	30
	4.5×10^{-3}	38	33
	5×10^{-3}	64	33
6-Phosphogluconate	1×10^{-4}	4	10

DISCUSSION

Homology and Divergent Specialization of PGI Loci

The formation of a heterodimeric enzyme by the association of subunits encoded by the two PGI loci provides strong evidence for their homology and hence for the origin of one of the loci through duplication. The well-known gene duplications for malate dehydrogenase, lactate dehydrogenase, and hemoglobins (Kitto and Kaplan, 1966; Fondy and Kaplan, 1965; Kaplan, 1965; Ingram, 1961) provide analogous examples. These genes also encode subunits which can associate to form functional proteins. Furthermore, the PGI homodimeric enzymes in *A. mexicanus* have certain similar kinetic properties such as Michaelis constants and reaction to inhibitors (phosphoenol pyruvate and 6-phosphogluconate). It is unlikely that the similarities of these molecules and their ability to form heterodimers (particularly in the heart) are the result of convergence in coding properties of nonhomologous genetic loci.

The evolutionary potential of duplicate genes was appreciated before molecular and biochemical techniques had fully documented their existence. Huxley's (1942) concept of divergent specialization of duplicate genetic loci giving great delicacy of adjustment to the genome was soon extended by Lewis (1951) and Stephens (1951) to a realization of the dual dynamic and conservative aspects of gene duplications. According to their view, gene duplications may initially release one locus from selection while the other maintains its previous function. The new locus is then free to undergo modifications through chromosomal rearrangements and mutations to assume new functions, frequently closely similar to those of the original gene. Our present views of gene duplications, supported with numerous examples and with a better understanding of the cellular mechanisms involved, represent only refinements of these original ideas (Watts and Watts, 1968*a*, Ohno, 1970).

The phosphoglucose isomerase enzymes encoded by the two independent genetic loci in *A. mexicanus* exemplify a modification and divergence in structure and function following gene duplication. The two enzymes, catalysing the same reaction, show striking differences in electrophoretic mobility, in heat inactivation properties, and probably also in reaction to the inhibitor adenosine 5′-triphosphate.

The specific pattern of tissue distribution is indirect evidence of biologically meaningful divergence in the PGI enzymes. PGI-2 is expressed very strongly in skeletal muscle but not elsewhere; the hybrid molecule is strongest in the heart; and PGI-1 predominates in most other tissues and organs. We are not yet in a position to discuss the physiological significance of the differential expression of subunits in the various tissues.

Evolutionary History of the PGI Gene Duplication in Osteichthyes

In an attempt to assess the extent of occurrence of two PGI loci in fish, with the ultimate aim of understanding the evolutionary history of the gene duplication and its time of origin, we have surveyed 53 species of bony fishes belonging to 38 families and 15 orders for the number of genetic loci encoding PGI. These species are listed in Table I, in natural or phyletic series as proposed by Bailey *et al.* (1970). In larger individuals of various species, the tissue distribution of the PGI enzymes was examined (Table I). In smaller specimens, the whole animal was ground for electrophoresis. Table I also indicates, for each species, whether any of the individuals examined were heterozygous at a PGI locus. The following discussion is based on Romer's (1966) phylogeny of bony fishes.

The duplicated gene for PGI is of widespread occurrence in fishes. For example, in the order Perciformes, which includes the great majority of spiny-rayed fishes (Lagler *et al.*, 1962), 19 of the 20 species examined belonging to 15 families unquestionably possess two PGI loci. Furthermore, in at least 38 of 46 species representing 34 families in the main line of teleost evolution (superorder Protacanthopterygii and derived forms), two loci are again present. Figure 7, modified from Romer (1966), summarizes the presumed family tree of higher bony fishes (Actinopterygii). A comparison of Fig. 7 and Table I confirms that representative species of all five superorders

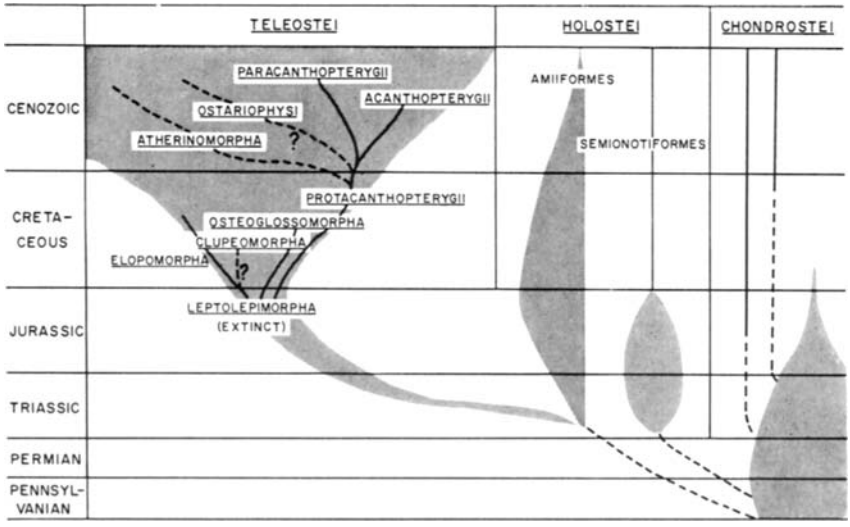

Fig. 7. "Family tree" of higher bony fishes (Actinopterygii). (Modified from Romer, 1966.)

of teleosts including and derived from the basal primitive stock Salmoniformes (Protacanthopterygii, Ostariophysi, Atherinomorpha, Paracanthopterygii, and Acanthopterygii) possess duplicated PGI loci. These five superorders include the great majority of all species of bony fishes and, for convenience, are specifically referred to as "higher teleosts" in the following discussion. [Recently, Nelson (1969) has reclassified the Protacanthopterygii, Paracanthopterygii, and Acanthopterygii into the cohort Euteleostei. A new superorder, Neoteleostei, now includes Myctophiformes, Paracanthopterygii, and Acanthopterygii. Or, according to Rosen and Patterson (1969), the Myctophiformes are elevated to superordinal status along with the Paracenthopterygii and Acanthopterygii. Since none of our conclusions are affected by these interpretations, we have chosen to retain Romer's classification until the matter is settled].

The patterns of tissue distribution of the PGI subunits of the higher teleost species and even their relative position on the gels show striking regularity. In all higher teleosts, PGI-2, always the slower-migrating, less anodal band, is invariably predominant in skeletal muscle, while the faster-migrating PGI-1 prevails in liver and other internal organs. The heart shows various concentrations of enzymes, but usually with the heterodimer or PGI-1 homodimer most strongly expressed. One or two satellite bands of very characteristic appearance consistently migrate slightly anodal to PGI-2.

In five of the 46 species of higher teleosts examined, evidence for genetic control of PGI by multiple loci is poor or lacking. In the lizard-fish and Atlantic midshipman, two distinct bands of similar mobility appear on gels, while in the bluefish and tonguefish only a single distinct band is present. The northern pipefish exhibits three bands very close in mobility, but since only a single specimen was available, the possibility that this phenotypic pattern results from heterozygosity at a single locus cannot be eliminated. The phenotypic patterns on the gels of these and other species clearly illustrate occasional difficulties of the electrophoretic technique. Two independent genetic loci encoding subunits of identical electrophoretic mobility would normally be scored as a single locus. The genetic basis of two or even three bands of similar mobility is likewise unclear, unless individual tissues are examined or occasional heterozygotes are seen. However, the following lines of evidence are advanced in favor of the proposal that these five species may also possess duplicate PGI loci along with all other higher teleosts: (1) the range of relative electrophoretic mobilities of PGI-1 and PGI-2 in other teleosts is considerable (great separation in some species, little in others), and, by chance, in certain species, we would expect PGI-1 and PGI-2 to exhibit identical or nearly identical mobilities; (2) the great majority of higher teleosts clearly exhibit two PGI loci, indicating early phylogenetic origin

of the gene duplication; and (3) in the case of the bluefish and tonguefish, numerous other closely related species (belonging to the same orders) have two PGI loci.

Therefore, while the possibility of secondary loss of a duplicate PGI locus in a few higher teleosts cannot be excluded, we conclude that the original gene duplication occurred sometime before the branching of the Protacanthopterygii and is now manifested in most if not all higher teleosts. Moreover, the striking similarity of tissue distribution and relative electrophoretic mobilities of the homodimers in all higher teleosts examined strongly suggests that the two loci had already diverged in structure and function in an ancestor of the Protacanthopterygii. In other words, in this early ancestor, PGI-1 may have already assumed its primary function in skeletal muscle and PGI-2 in the other internal organs.

The Protacanthopterygii (from which probably arose all higher teleost orders), appear to have their origin in primitive salmon-like fish of the Upper Cretaceous, and in the pike (*Esox*) of the early Tertiary. However, by the Lower Cretaceous, at least three other primitive types of teleosts, giving rise to independent lines represented by several living forms, had also made their appearance. These were the superorders Elopomorpha, Osteoglossomorpha, and Clupeomorpha, comprising the remainder of the living teleosts.

The superorder Elopomorpha is today represented by the tarpon group of fishes and by eels (Anguilliformes). The only member of Elopomorpha examined in this study was the yellow conger, *C. flava*. Again, two PGI loci are demonstrable, but the pattern of tissue distribution appears to differ from that of higher teleosts. Skeletal muscle shows approximately equal concentrations of subunits encoded by each locus, as do the eggs, while heart shows only the PGI-1 homodimer. The heterodimer band is very weak in these tissues. Living representatives of the tropical superorder Osteoglossomorpha were not obtained for our studies.

Far more successful in modern times are the Clupeiformes, living representatives of a group, Clupeomorpha, whose origin is still uncertain. Early herring-like ancestors were well established in the Lower Cretaceous, but whether they arose independently from an ancestral teleost stock or are an offshoot of the elopomorphs is unknown. Three species of Clupeidae (menhaden, herring, and gizzard shad) show similar patterns on the gels. In each case, a single band stains intensely, with much lighter, slightly anodal satellite bands. This pattern appears similar to the typical banding pattern of the PGI-2 enzymes in all other teleosts. In addition, thin light bands, unlike the typical PGI-1 band of other teleosts, appear far anodal and cathodal to the intense band. Phenotypes of various tissues of the gizzard shad are closely similar. Whether these results are evidence for a single PGI locus or whether multiple loci are present which encode non-tissue-specific enzymes is un-

certain. In either case, however, it appears that, relative to other teleosts, the PGI enzymes in the Clupeidae are under a different type of genetic control.

All species discussed to this point belong to the Teleostei, an assemblage that began flourishing in the Cretaceous and includes the great majority of living fishes. However, in the Jurassic and Lower Cretaceous, another group of more primitive bony fishes, the holosteans, were dominant. Although holosteans were almost completely replaced by the teleosts in the Upper Cretaceous, two genera survived and have representatives living today—the gars and bowfins. The bowfin (*Amia calva*) traces its ancestry back to the Triassic, where the Amiiformes diverged from the line leading to the teleosts (Fig. 7). The Semionotiformes, represented only by the living gars, probably diverged still earlier from a primitive stock, perhaps in the late Paleozoic.

The bowfin and two species of gar were examined for PGI activity. In each case, a single intense band plus the characteristic anodal secondary bands appear on the gel, in extracts both from whole animals and from various tissues. Thus there is no good evidence for more than a single genetic locus encoding PGI.

In summarizing this section, we may note that there are general trends observable from Table I which warrant an estimate of the time of origin of the gene duplication and a consideration of the general history of the divergent specialization of the PGI homodimers into various tissues. Assuming that holosteans have only a single PGI locus and have not secondarily lost a duplicate gene, the gene duplication must have arisen sometime after the phylogenetic branching of the holosteans and before the branching of the higher teleosts from the Elopomorpha. A likely candidate for this origin is Leptolepiformes, a generalized group prominent in the Jurassic which gave rise to all modern teleosts. Furthermore, the enzymes probably diverged in function (as evidenced by tissue specificities) very early in the history of teleosts, although probably after the Clupeiformes had branched from ances ral stock. It should be most interesting to study representatives of the Osteoglossomorpha to test these hypotheses. The chondrosteans, which were the precursors of the holosteans and are represented by two living American families, the sturgeons (Acipenseridae) and paddlefishes (Polyodontidae), also remain to be examined.

Gene Duplication Through Polyploidization

Gene duplications may arise through various mechanisms of tandem duplication involving only small portions of the genome or through polyploidization. Ohno (1970) and Ohno *et al.* (1968) view the two mechanisms as being complementary and further feel that, since polyploid evolution is denied to mammals, birds, and reptiles, due to their well-established chromosomal

sex determining mechanisms, most successful achievements of gene duplications in vertebrates must have taken place at the stage of fishes and amphibians.

Multiple loci, presumably resulting from duplications of genes encoding a wide variety of proteins, have previously been reported in fishes: 6-phosphogluconate dehydrogenase in certain cyprinids (Klose *et al.*, 1969; Bender and Ohno, 1968), hemoglobins in hagfish and salmonids (Ohno and Morrison, 1966; Tsuyuki and Gadd, 1963), malate dehydrogenase in salmonids (Bailey *et al.*, 1969, 1970), lactate dehydrogenase in cyprinids and salmonids (Klose

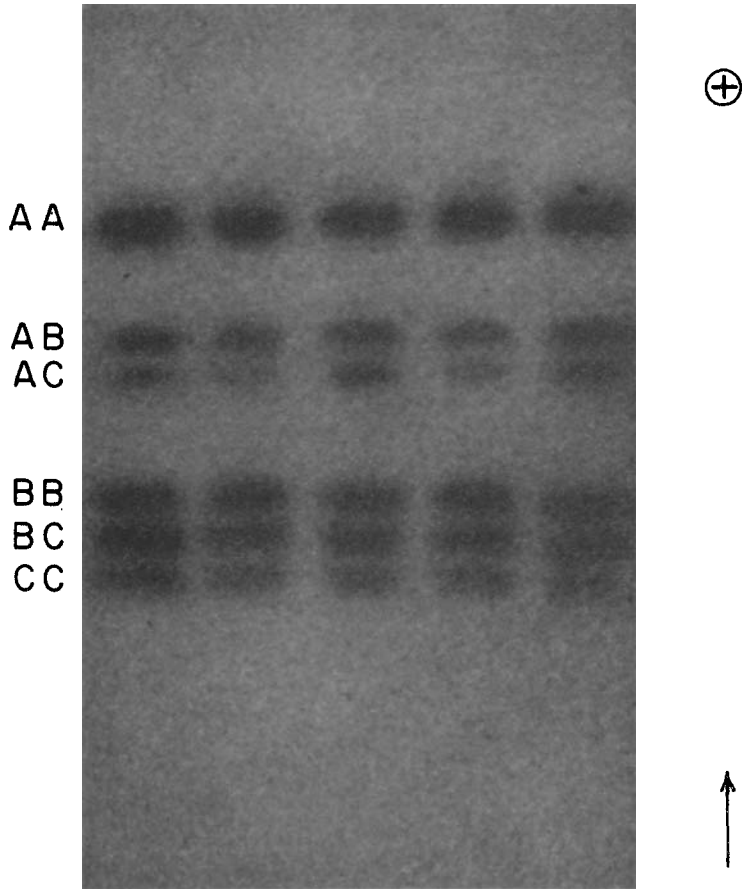

Fig. 8. Phenotypes of phosphoglucose isomerases from whole-animal extracts of brook trout. All individuals are presumably homozygous at three PGI loci. A, B, and C: Subunits encoded by *Pgi-1*, *Pgi-2*, and *Pgi-3*, respectively.

et al., 1968, 1969; Massaro and Markert, 1968; Morrison and Wright, 1966), and isocitrate dehydrogenase in certain cyprinids (Quiroz-Gutierrez and Ohno, 1970). Several of these gene duplications are apparently restricted to a few salmonids and cyprinids which, from cytological and DNA evidence, are probably tetraploid (Ohno *et al.*, 1967; Massaro and Markert, 1968; Ohno, 1970).

Three presumably tetraploid salmonid species (brook trout, brown trout, and rainbow trout) were examined for PGI activity. All individuals exhibit six bands, most likely representing random association of subunits encoded by three genetic loci (Fig. 8). Apparently, at least one of the original pair of PGI loci has reduplicated to form a third locus as a result of polyploidization. Furthermore, PGI-3 (the homodimer produced by the third locus) resembles PGI-2 in tissue distribution (predominant in muscle, weak elsewhere) and in electrophoretic mobility. Hence, as a result of polyploidization, two former *Pgi-2* locus alleles have been incorporated into the genome. However, in salmonids, the *Pgi-1* locus shows no evidence of having reduplicated. Perhaps alleles of the *Pgi-1* locus incorporated into the genome are identical, and thus the organism simply has more loci making PGI-1, or perhaps the duplicated *Pgi-1* locus did not confer a selective advantage on the organism and thus was silenced.

In tetraploid goldfish (Cyprinidae), six major bands are present in all individuals, again giving evidence for three genetic loci. In this case, duplication of the *Pgi-1* locus through polyploidization has resulted in a new locus encoding a homodimer (PGI-4) which, along with PGI-1 and their heterodimer, predominates in liver and other organs and is very weak in skeletal muscle. The heart expresses PGI-1, PGI-2, PGI-4, and their heterodimers, although the PGI-1–PGI-4 complex is strongest. PGI-2 is, as usual, strongest in muscle.

Tetraploid trout and goldfish demonstrate that new genetic loci can be incorporated into the genome through polyploidization, although not all genetic loci give rise to new loci detectable by electrophoresis. Unfortunately, we do not know whether the original PGI gene duplication in fishes arose through tandem duplication or through polyploidization.

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REFERENCES

Avise, J. C., and Selander, R. K. (1972). Evolutionary genetics of cave-dwelling fishes of the genus *Astyanax*. *Evolution* **26**:1.

Bailey, G. S., Cock, G. T., and Wilson, A. C. (1969). Gene duplication in fishes: Malate dehydrogenase of salmon and trout. *Biochem. Biophys. Res. Commun.* **34**:605.

Bailey, G. S., Wilson, A. C., Halver, J. E., and Johnson, C. L. (1970). Multiple forms of supernatant malate dehydrogenase in salmonid fishes. *J. Biol. Chem.* **245**:5927.

Bailey, R. M., Fitch, J. E., Herald, E. S., Lachner, E. A., Lindsey, C. C., Robins, C. R., and Scott, W. B. (1970). A list of common and scientific names of fishes from the United States and Canada. *Am. Fish. Soc. Serial. Publ.*, No. 6.

Bender, K., and Ohno, S. (1968). Duplication of the autosomally inherited 6-phosphogluconate dehydrogenase gene locus in tetraploid species of cyprinid fish. *Biochem. Genet.* **2**:101.

Carter, N. D., and Parr, C. W. (1967). Isoenzymes of phosphoglucose isomerase in mice. *Nature* **216**:511.

DeLorenzo, R. J., and Ruddle, F. H. (1969). Genetic control of two electrophoretic variants of glucose phosphate isomerase in the mouse (*Mus musculus*). *Biochem. Genet.* **3**:151.

Detter, J. C., Ways, P. O., Giblett, E. R., Baughman, M. A., Hopkinson, D. A., Povey, S., and Harris, H. (1968). Inherited variations in human phosphohexose isomerase. *Ann. Hum. Genet.* **31**:329.

Fondy, T. P., and Kaplan, N. O. (1965). Structural and functional properties of the H and M subunits of lactic dehydrogenases. *Ann. N.Y. Acad. Sci.* **119**:888.

Hines, M. C., and Wolfe, R. G. (1963). Phosphoglucose isomerase. II. Influence of *p*H on kinetic parameters. *Biochemistry* **2**:770.

Huxley, J. (1942). *Evolution: The Modern Synthesis*, Allen and Unwin, London.

Ingram, V. M. (1961). Gene evolution and the haemoglobins. *Nature* **189**:704.

Johnson, W. E., and Selander, R. K. (1971). Protein variation and systematics in kangaroo rats (genus *Dipodomys*). *Syst. Zool.* **20**: 377.

Johnson, W. E., Selander, R. K., Smith, M. H., and Kim, Y. J. (1972). Biochemical genetics of sibling species of the cotton rat (*Sigmodon*). *Stud. Genet. (Univ. Texas Publ.)* **7** (7213):297.

Kahana, S. E., Lowry, O. H., Schulz, D. W., Passonneau, J. V., and Crawford, E. J. (1960). The kinetics of phosphoglucoisomerase. *J. Biol. Chem.* **235**: 2178.

Kaplan, N. O. (1965). Evolution of dehydrogenases. In Bryson, V., and Vogel, H. J. (eds.), *Evolving Genes and Proteins*, Academic Press, New York, p.243.

Kitto, G. B., and Kaplan, N. O. (1966). Purification and properties of chicken heart mitochondrial and supernatant malic dehydrogenases. *Biochemistry* **5**:3966.

Klose, J., Wolf, U., Hitzeroth, H., Ritter, H., Atkin, N. B., and Ohno, S. (1968). Duplication of LDH gene loci by polyploidization in the fish order Clupeiformes. *Humangenetik* **5**:190.

Klose, J., Wolf, U., Hitzeroth, H., Ritter, H., and Ohno, S. (1969). Polyploidization in the fish family Cyprinidae, order Cypriniformes. II. Duplication of the gene loci coding for lactate dehydrogenase (E.C.:1.1.1.27) and 6-phosphogluconate dehydrogenase (E.C.:1.1.1.44) in various species of Cyprinidae. *Humangenetik* **7**:245.

Lagler, K. F., Bardach, J. E., and Miller, R. R. (1962). *Ichthyology*, Wiley, New York.

Lewis, E. B. (1951). Pseudoallelism and gene evolution. *Cold Spring Harbor Symp. Quant. Biol.* **16**:159.

Massaro, E. J., and Markert, C. L. (1968). Isozyme patterns of salmonoid fishes: Evidences for multiple cistrons for lactate dehydrogenase polypeptides. *J. Exptl. Zool.* **168**: 223.

Morrison, W. J., and Wright, J. E. (1966). Genetic analysis of three lactate dehydrogenase isozyme system in trout: Evidence for linkage for genes coding subunits A and B. *J. Exptl. Zool.* **163**:259.

Nakagawa, Y., and Noltmann, E. A. (1967). Multiple forms of yeast phosphoglucose isomerase. I. Resolution of the crystalline enzyme into three isoenzymes. *J. Biol. Chem.* **20**:4782.

Nelson, G. J. (1969). Gill arches and the phylogeny of fishes, with notes on the classification of vertebrates. *Bull. Am. Mus. Nat. Hist.* **141**:475.

Noltmann, E. A. (1964). Isolation of crystalline phosphoglucose isomerase from rabbit muscle. *J. Biol. Chem.* **239**:1545.

Nottebohm, F., and Selander, R. K. (1972). Vocal dialects and gene frequencies in the Chingolo sparrow (*Zonotrichia capensis*). *Condor* **74**:137.
Ohno, S. (1970). *Evolution by Gene Duplication*, Springer-Verlag, New York.
Ohno, S., and Morrison, M. (1966). Multiple gene loci for the monomeric hemoglobin of the hagfish (*Eptatretus stoutii*). *Science* **154**:1034.
Ohno, S., Muramoto, J., Christian, L., and Atkin, N. B. (1967). Diploid–tetraploid relationship among Old-World members of the fish family Cyprinidae. *Chromosoma* **23**:1.
Ohno, S., Wolf, U., and Atkin, N. B. (1968). Evolution from fish to mammals by gene duplication. *Hereditas* **59**:169.
Prasad, R., and Wright, D. A. (1971). Sponge method of preparative electrophoresis. *Isozyme Bull.* **4**:12.
Quiroz-Gutierrez, A., and Ohno, S. (1970). The evidence of gene duplication for S-form NADP-linked isocitrate dehydrogenase in carp and goldfish. *Biochem. Genet.* **4**:93.
Ralin, D. B., Selander, R. K., and Yang, S. Y. (1972). Protein variation and systematics in the genus *Hyla*. In preparation.
Ramsey, P. R., Avise, J. C., Smith, M. H., and Urbston, D. F. (1972). Protein variation and genic heterozygosity in white-tailed deer (*Odocoileus virginianus*). In preparation.
Romer, A. S. (1966). *Vertebrate Paleontology*, 3rd ed., University of Chicago Press, Chicago.
Rosen, D. E., and Patterson, C. (1969). The structure and relationships of the paracanthopterygian fishes. *Bull. Am. Mus. Nat. Hist.* **141**: 357.
Selander, R. K., Hunt, W. G., and Yang, S. Y. (1969). Protein polymorphism and genic heterozygosity in two European subspecies of the house mouse. *Evolution* **23**:379.
Selander, R. K., Yang, S. R., Lewontin, R. C., and Johnson, W. E. (1970). Genetic variation in the horseshoe crab (*Limulus polyphemus*), a phylogenetic "relic." *Evolution* **24**:402.
Selander, R. K., Smith, M. H., Yang, S. Y., Johnson, W. E., and Gentry, J. B. (1971). Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). *Stud. Genet.* (*Univ. Texas Publ.*) **6 (7103)**:49.
Selander, R. K., Johnson, W. E., and Avise, J. C. (1972). Biochemical population genetics of fiddler crabs (*Uca*). In preparation.
Stephens, S. G. (1951). Possible significance of duplication in evolution. *Advan. Genet.* **4**:247.
Tsuyuki, H., and Gadd, R. E. A. (1963). The multiple hemoglobins of some members of the Salmonidae family. *Biochim. Biophys. Acta* **71**:219.
Watts, R. L., and Watts, D. C. (1968*a*). Gene duplication and the evolution of enzymes. *Nature* **217**:1125.
Watts, R. L., and Watts, D. C. (1968*b*). The implications for molecular evolution of possible mechanisms of primary gene duplication. *J. Theoret. Biol.* **20**:227.
Webster, T. P., Selander, R. K., and Yang, S. Y. (1972). Genetic variability and similarity in the *Anolis* lizards of Bimini. *Evolution* (submitted).
Whitt, G. S. (1970). Genetic variation of supernatant and mitochondrial malate dehydrogenase isozymes in the teleost *Fundulus heteroclitus*. *Experientia* **26**:734.
Yoshida, A., and Carter, N. D. (1969). Nature of rabbit phosphoglucose isomerase isozymes. *Biochim. Biophys. Acta* **194**:151.