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REVIEW

Nongenetic Variation, Genetic-Environmental Interactions and Altered Gene Expression. III. Posttranslational Modifications

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ABSTRACT. The use of protein electrophoretic data for determining the relationships among species or populations is widespread and generally accepted. However, posttranslational modifications have been discovered in many of the commonly analyzed proteins and enzymes. Posttranslational modifications often alter the electrophoretic mobility of the modified enzyme or protein. Because posttranslational modifications may affect only a fraction of the total enzyme or protein, an additional staining band often appears on gels as a result, and this may confound interpretations. Deamidation, acteylation, proteolytic modification, and oxidation of sulfhydryl groups are modifications that often result in an electrophoretic mobility shift. Sialic acid-induced heterogeneity has been documented for many enzymes, but neuraminidase treatment can often remove sialic acids and produce gel patterns that are easier to interpret. In some cases, ontogenetic and tissue-specific expression may be due to posttranslational modifications rather than gene control and restricted expression, respectively. Methods of preventing, detecting and eliminating posttranslational modifications are discussed. Some posttranslational modifications may be useful for detecting cryptic genetic polymorphisms. COMP BIOCHEM PHYSIOL 118A;3:551–572, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Allozymes, acclimation, polymorphism, heterogeneity, secondary isozymes, acetylcholinesterase, transferrin, esterases

INTRODUCTION

Protein electrophoretic techniques have been and continue to be widely used and accepted tools in systematic and population studies of vertebrates and invertebrates (6,27, 68,119,165,262,277,283). However, there are many factors that can affect the results of an electrophoretic study. Some investigators discussed briefly the presence of "extra" proteins which were posttranslational modifications or nongenetic in origin; the possible ramifications of such variation with regard to systematics has rarely been considered (21,26,130,152,153,177,187,268). Murphy *et al.* (178) discussed causes of posttranslational modifications (both genetic and nongenetic), cautioned against their occurrence and suggested ways of avoiding and detecting such variation. Finnerty and Johnson (71) and Johnson *et al.* (120) determined that some "electromorphs" of xanthine dehydrogenase (EC 1.1.1.204) and aldehyde oxidase (EC 1.2.3.1) were actually posttranslational modifications due to at least two modifier loci; they also provided an in-depth discussion of the potential impact of such phenomena on levels of polymorphisms. Titus (255) investigated the use of recently road-killed vs live-caught amphibians and observed anodal, secondary isozymes for nine commonly used enzymes. The secondary isozymes were present in both road-killed and live-caught samples and "made the scoring of polymorphisms difficult at times . . ." [(255), p. 15].

The number of isozymes or allozymes (= multiple staining bands on a gel) expressed has been shown to be affected by temperature, diet, pH, photoperiod, sex, female reproductive state, posttranslational changes, sample processing procedures, storage time, pollution, disease, parasites, and other stressors [see (193,194)]. Misinterpretation of nongenetic variation as true, genetic polymorphisms and the effects the changes will have with regard to systematic studies have not been treated thoroughly. The purpose of this review is to examine known posttranslational modifications of

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enzymes and proteins that result in altered electrophoretic mobility and discuss methods that will assist in detecting and eliminating such problems.

A number of investigators have discussed nomenclature pertaining to isozymes (139,161,165,173,185,214,277,278, 285). Rothe (214) provided the most detailed discussion of isozyme nomenclature and considered isozymes formed by indirect genetic mechanisms to be secondary isozymes, and this term will be used herein due to both its generality in meaning and its prior usage in earlier literature [e.g., (73,227,255)].

Abbreviations

Abbreviations for enzymes generally follow Shaklee *et al.* (225) and Murphy *et al.* (178), and standard names and Enzyme Commission numbers follow the International Union of Biochemistry and Molecular Biology (115): acetylcholinesterase (AChE), EC 3.1.1.7; acid phosphatase (ACP), EC 3.1.3.2; alkaline phosphatase (ALP), EC 3.1.3.1; α-amylase (AMY), EC 3.2.1.1; butyrylcholinesterase (BChE), EC 3.1.1.8; catalase (CAT), EC 1.11.1.6; creatine kinase (CK), EC 2.7.3.2; cholinesterase (ChE) (AChE and/or BChE?), EC 3.1.1._; cytosol aminopeptidase (CAP), EC 3.4.11.1 (formerly leucine aminopeptidase); esterases (EST), EC 3.1.1._; glucose-6-phosphate 1-dehydrogenase (G6PDH), EC 1.1.1.49; glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (GAPDH), EC 1.2.1.12; glycerol-3-phosphate dehydrogenase (NAD^+) (G3PDH), EC 1.1.1.8; glucose-6-phosphate isomerase (GPI), EC 5.3.1.9; hemoglobin (HB); lactate dehydrogenase (LDH), EC 1.1.1.27; malate dehydrogenase (MDH), EC 1.1.1.37; exo-α-sialidase (commonly referred to as neuraminidase or sialidase) (NEU), EC 3.2.1.18; phosphoglucomutase (PGM), EC 5.4.2.2; transferrin, TF. Other abbreviations: sialic acid (*N*-acetyl neuraminic acid) (Sia), ethylenediaminetetraacetate (EDTA).

POSTTRANSLATIONAL MODIFICATION

In Vivo *Modifications*

Of the many ways in which the structure of a polypeptide can be modified, changes in its primary structure are probably most important (264). Some of these alterations may render the protein completely or partially inactive, while others may enhance the activity under certain conditions. Uy and Wold (264) listed a total of 140 possible amino acid forms, which included the 20 "primary" amino acids plus forms derived from phosphorylations, deamidations, acetylations, and methylations to name just a few. Wold (288) and Harding (92) reviewed *in vivo* posttranslational modifications of proteins, and should be referred to concerning posttranslational modifications that have not been discussed herein. There are additional compilations a variety of posttranslational modifications to which the reader may refer (90,93,118,259,290,291) and specifically for posttranslational modifications involving lipids (33,220).

There are a number of mechanisms by which multiple products arise from a single gene [compiled from (22,52, 145,216,217,288,289)]:

Transcriptional
- —Alternate transcription (>1 pre-mRNA transcribed)

Posttranscriptional
- —Alternative splicing (different mRNAs produced from 1 pre-mRNA)
- —Alternate translation (>1 polypeptide translated from 1 mRNA)
- —Cotranslational (amino acids modified on polypeptide before release from polysome)
- —Posttranslational (polypeptide modified after release from polysome)

Uy and Wold [(264), p. 894] stated that ". . . only a fraction of the molecules of one kind are modified.", and Dykhuizen *et al.* (61) reported this for *Escherichia coli* ALP. Most of the posttranslational modifications discussed herein can produce at least one additional staining fraction [e.g., (60, 89,92)]. Therefore, two or more forms of the enzyme may be present, one being the original, transcribed product and the other being a modified product. If these different forms of an enzyme are all catalytically active, they may appear on gels as multiple bands, indicating the presence of several "isozymes" or "allozymes" when, in fact, there is only one enzyme. The formation of isozymes due to *in vivo* modifications adds yet another possible cause of variation which may be falsely construed as being genetic in origin (74,178,187). Five of the 20 "primary" amino acids are charged and useful in that they reveal genetic differences through charge differences. The five charged amino acids are lysine (+), arginine (+), histidine (+), aspartic acid (−) and glutamic acid (−). Proteins with a net positive charge migrate toward the negative (cathodal) pole during gel electrophoresis, while proteins with a net negative charge move toward the positive (anodal) pole (261). Posttranslational modifications can alter the charge of the genetically-encoded amino acid and either mask genetic differences or produce apparent differences. In *Drosophila melanogaster*, superoxide dismutase (EC 1.15.1.1) allozymes differing by only one amino acid (lysine (+) vs aspartic acid (−) or asparagine (n)) exhibited different electrophoretic mobilities, while other superoxide dismutase allozymes differing by two substitutions (histidine (+) and proline (n) vs serine (n) and either glutamic acid (−) or glutamine (n)) were electrophoretically identical (143). Cobbs and Prakash (42) also mentioned several cases where a single amino acid substitution resulted in a change in electrophoretic mobility. Many of the posttranslational modifications listed in Table 1 occur in the charged amino acids and the α-NH_2 group, and the listed enzymes are routinely used in taxonomic, systematic, and population genetic research. Modified enzymes may remain cryptic with

TABLE 1. Commonly used enzymes or proteins that are known to be modified by some posttranscriptional process (reversible and irreversible). Many of the cited cases result in a change in electrophoretic mobility

Enzyme/protein	Modification	Reference(s)
Aspartate aminotransferase (EC 2.6.1.1)	deamidation	117, 208, 286
	carbamylation	189
Acetylcholinesterase (EC 3.1.1.7)	aggregation/dissociation	136
	proteolytic modification	146
Acid phosphatase (EC 3.1.3.2)	sulfhydryl oxidation	110, in 214
	carbamylation	189
Adenosine deaminase (EC 3.5.4.4)	sulfhydryl oxidation	110, 133, in 214
	aggregation/dissociation	in 214
	carbamylation	189
Alcohol dehydrogenase (EC 1.1.1.1)	N^{α}-acetylation	20
	acetylation	257
	deamidation	208
	cofactor binding	116, 260
Adenylate kinase (EC 2.7.4.3)	N^{α}-acetylation	20
	acetylation	257
Alanine aminotransferase (EC 2.6.1.2)	carbamylation	189
Alkaline phosphatase (EC 3.1.3.1)	acetylation	175
	proteolytic degradation	61, 221
	immunoglobulin-conjugation	29
	aggregation/dissociation	in 214
	phosphorylation	49
α-amylase (EC 3.2.1.1)	glycosylation	126
	glycogen binding	251
	deamidation	126, 147, in 169, 208
	immunoglobulin-conjugation	in 244
	methylation	188
	N^{α}-acetylation	in 288
Butyrylcholinesterase (EC 3.1.1.8)	albumin-conjugation	in 36
Carbonate dehydratase (EC 4.2.1.1)	glycation	252
	deamidation	77, 208
	acetylation	257
	N^{α}-acetylation	20
	aggregation/dissociation	in 214
Catalase (EC 1.11.1.6)	deamidation	208
	sulfhydryl oxidation	1, 109
Creatine kinase (EC 2.7.3.2)	proteolytic modification	in 244
	immunoglobulin-conjugation	37
	phosphorylation	202
Citrate (*si*)-synthase (EC 4.1.3.7)	methylation	188
Cytochrome c (EC 4.4.3.__)	deamidation	74, 208
	N^{α}-acetylation	20
	methylation	188
Cytosol aminopeptidase (EC 3.4.11.1)	carbamylation	189
Enolase (EC 4.2.1.11)	phosphorylation	46, 47, 181
	acetylation	257
	deamidation	208
	N^{α}-acetylation	in 288
	carbamylation	189
Esterase (EC 3.1.1.__)	deamidation	208
	aggregation/dissociation	62, in 214
	carbamylation	189
Fructose-bisphosphate aldolase (EC 4.1.2.13)	deamidation	137, 168, 208
	proteolytic modification	111, in 142
Fructose bisphosphatase (EC 3.1.3.11)	proteolytic modification	192, 195–197
	N^{α}-acetylation	20
Fumarate hydratase (EC 4.2.1.2)	deamidation	208
Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12)	N^{α}-acetylation	20
	acetylation	257
	deamidation	208
	carbamylation	189
Glutamate dehydrogenase (EC 1.4.1.__)	acetylation	257
	aggregation/dissociation	in 214
Glycerol-3-phosphate dehydrogenase(NAD^+) (EC 1.1.1.8)	acetylation?	269
	deamidation	in 18, 208
	proteolytic modification	in 18

TABLE 1. (*Continued*)

Enzyme/protein	Modification	Reference(s)
Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	deamidation	208, 231
	cofactor binding	10, 35
	aggregation/dissociation	131, 295
	protein-conjugation	131
Glucose-6-phosphate isomerase (EC 5.3.1.9)	acetylation	257
	N^{α}-acetylation	in 88
	proteolytic modification	in 88
	aggregation/dissociation	in 88
	sulfhydryl oxidation	87, in 88, 182, in 214
	carbamylation	189
Hemoglobin	glycation	45, in 92, 157
	acetylation	157
	N^{α}-acetylation	20, 25, 92, 172, 200
	carbamylation	92
	oxidation	243
	aggregation/dissociation	132, 199, 204, 243
	proteolytic modification	132
	Haptoglobin-conjugation	186
Hemoglobin (Providence)	deamidation	172
Hexokinase (EC 2.7.1.1)	sulfhydryl oxidation	in 214
	proteolytic modification	in 214
	aggregation/dissociation	in 214
	Ubiquitin-conjugation	156
Lactate dehydrogenase (EC 1.1.1.27)	deamidation	99, 208, 287
	phosphorylation	46, 47
	immunoglobulin-conjugation	in 244
	sulfhydryl oxidation	165, in 214
	cofactor binding	40, 285
	conformational (?)	203, 205
Malate dehydrogenase (EC 1.1.1.37)	deamidation	208
	aggregation/dissociation	in 214
	carbamylation	189
Malate dehydrogenase (decarboxylating) (EC 1.1.1.39)	aggregation/dissociation	in 214
Nucleoside-triphosphate pyrophosphatase (EC 3.6.1.19)	sulfhydryl oxidation	110
	N^{α}-acetylation	20
Peptidase (EC 3.4.11.__)	sulfhydryl oxidation	110
Peroxidase (EC 1.11.1.7)	deamidation	208
Phosphoglycerate mutase (EC 5.4.2.1)	phosphorylation	46
	carbamylation	189
Phosphogluconate dehydrogenase (EC 1.2.1.44)	carbamylation	189
Phosphoglycerate kinase (EC 2.7.2.3)	acetylation	257
	carbamylation	189
Phosphoglucomutase (EC 5.4.2.2)	sulfhydryl oxidation	53, 54, 73, 110
	deamidation	208
	carbamylation	189
Pyruvate kinase (EC 2.7.1.40)	acetylation	257
	deamidation	208
	sulfhydryl oxidation	8
	proteolytic modification	in 214
Plasminogen	deamidation	208
Superoxide dismutase (EC 1.15.1.1)	glycosylation	254
	glycation	252
	N^{α}-acetylation	20
Succinate-CoA ligase (EC 6.2.1.4-5)	phosphorylation	7
	sulfhydryl oxidation	7
Tyrosine transaminase (EC 2.6.1.5)	phosphorylation	94
	N^{α}-acetylation	94
	proteolytic modification	94
	carbamylation	122
Transferrin	deamidation	208
Triose-phosphate isomerase (EC 5.3.1.1)	deamidation	in 88, 231, 296
	carbamylation	189
Urease (EC 3.5.1.5)	sulfhydryl oxidation	72
	aggregation/dissociation	72
X-pro dipeptidase (Peptidase D) (EC 3.4.13.9)	sulfhydryl oxidation	110

one technique and be detected by another. For example, fructose-bisphosphate aldolase (EC 4.1.2.13) and G6PDH appeared unchanged by gel electrophoresis, but isoelectric focusing discriminated between primary and secondary isozymes (60). Some protein modifications are enzyme-catalyzed, while others are not (264,290,291).

Kaplan (124) and Shaw (227) provided the earliest discussions of "nongenetic isozymes." Deamidation, acetylation, proteolytic modification, aggregation, changes in conformation, and differences in bound molecules were cited as some of the known factors involved with the formation of secondary isozymes (Table 1). Rothe (214) produced an excellent review entitled, "A Survey on the Formation and Localization of Secondary Isozymes in Mammalia," which discussed the posttranslational formation of "isozymes." Rothe (214) examined nine causes of secondary isozyme formation including: aggregation and polymerization, oxidation/reduction, proteolytic modification, variation in carbohydrate groups, deamidation, aggregation of substrates and cosubstrates, temperature, pH, and conformational changes. Some of the cases from Rothe (214) were included here; however, it is recommended highly that the reader refer to Rothe (214) for additional information and references. Webster and Murphy (280) cited several literature reports of posttranslational modification as the cause of secondary isozymes (e.g., GAPDH and triose-phosphate isomerase, EC 5.3.1.1). Murphy *et al.* (178) provided examples of posttranslational modification on zymograms for GPI and MDH (their Figs. 14 and 17, respectively). Posttranslational modification was considered to be involved in mannose-6-phosphate isomerase (EC 5.3.1.8) variation observed in kidney and muscle tissue of *Crotalus v. viridis* (177).

Deamidation

Deamidation (Asn→Asp or Gln→Glu) is a common posttranslational modification (287) that can alter the charge and possibly migration of a polypeptide in a gel matrix. Deamidation can occur both enzymatically and non-enzymatically (208). Additional AMY isozymes observed in chickens were considered posttranslational modifications of AMY_2 and AMY_3, and deamidation of asparagine or glutamine residues was thought to be the probable mechanism for the additional isozymes (144). Karn *et al.* (126,127) studied human AMY and identified the processes of deamidation and glycosylation/deglycosylation as the causes of posttranslational modifications of this enzyme. The deamidation of two Asn residues in LDH B_4 may be responsible for its ability to function as a structural protein in eye lens rather than its normal catalytic function, even though both are encoded by only one gene (99). Eye lens enzymes are relatively long-lived since the cell nuclei are lost following differentiation into fibers; therefore, the enzymes may be more prone to posttranslational modifications during the course of their relatively long existence. Several other enzymes, G6PDH, triose-phosphate isomerase and nucleoside-triphosphate pyrophosphatase (EC 3.6.1.19), in eye lens and other tissues exhibit anodic secondary isozymes attributed to deamidation (231,296). Also, new TF isoforms appear as a result of deamidation (207). Deamidation is accelerated by ascorbic acid and O_2, and deamidation rate is also dependent on pH and neighboring residues in the polypeptide (207,208).

Acetylation

Allfrey *et al.* (5) indicated that acetylation of a lysine ε-amino group (resulting in the formation of ε-*N*-Acetyllysine) will neutralize its positive charge, and the electrophoretic mobility of histone H_4 varies according to the number of acetylated lysine residues. Acetylation of HB may be responsible for observed heterogeneity in some cases (132), and acetylation of the *N*-terminal amino acid, which normally contributes a positive charge, results in a neutral charge. Such a change to one chain type in HB would result in a loss of two positive charges and likely result in a mobility difference in gel electrophoresis. Some human HB have acetylated *N*-termini (172). Three fish species from three families (Salmonidae, Cyprinidae, and Catostomidae) and *Rana catesbeiana* tadpoles have α chain acetylation of the *N*-terminal serine residue (25,163,200). Commonly analyzed enzymes known to be *N*-terminally acetylated in some taxa are GAPDH, GPI, phosphoglycerate kinase (EC 2.7.2.3), glutamate dehydrogenase (EC 1.4.1._), alcohol dehydrogenase (EC 1.1.1.1), carbonate dehydratase (EC 4.2.1.1), adenylate kinase (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40), enolase (EC 4.2.1.11), fructose bisphosphatase (EC 3.1.3.11), and superoxide dismutase (257). Moss and Thomas (175) experimentally acetylated ALP isozymes *in vitro*, and electrophoretic mobility was altered following acetylation. *N*-terminal acetylations are catalyzed by amino-terminal acetyltransferases and can occur either cotranslationally or posttranslationally (258). Acetylation may be involved in the ubiquitin-protein degradation scheme (102).

Proteolytic Modification

Secondary isozymes of CK, HB and fructose bisphosphatase can be the result of peptidase cleavage of terminal residues (64,132,192,195–197,244). Wright *et al.* (294) discussed apparent proteolytic modification of LDH B from pyloric caeca and intestine of rainbow trout. Active BChE monomers, dimers, and trimers in human serum were considered degradation products of tetramers resulting from proteolytic modification (146). Some carboxypeptidase activity can be reduced or eliminated with EDTA or β-mercaptoethanol (63).

Phosphorylation and Carbamylation

Phosphorylation of CK subunits resulted in electrophoretic heterogeneity and also affected the apparent Michaelis-

Menten constant for phosphocreatine (202). Phosphorylation of histone H_1 alters the electrophoretic mobility and complicates gel pattern interpretation (166). Carbamylation can alter electrophoretic mobility of enzymes and proteins. Papayannopoulou *et al.* (189) examined the effects of *in vitro* and *in vivo* cyanate treatment on electrophoretic mobility of 25 enzymes and HB in red cells and the same enzymes in brain, liver, kidney, and muscle. All 25 enzymes were carbamylated *in vitro*, while only 15 of 25 were affected by *in vivo* treatments (ACP, CAP, EST, GPI, GAPDH, MDH, PGM, phosphogluconate dehydrogenase (decarboxylating, EC 1.1.1.44), triose-phosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase (EC 5.4.2.1), aspartate aminotransferase (EC 2.6.1.1), alanine aminotransferase (EC 2.6.1.2), adenosine deaminase (EC 3.5.4.4), enolase as determined by the appearance of new bands of activity with increased anodal migration on starch gels. Johnson *et al.* (122) presented evidence that 2 of 3 tyrosine transaminase (EC 2.6.1.5) isozymes from rat liver were secondary isozymes, and Hargrove and Granner (94) found that tyrosine transaminase may modified by phosphorylation, *N*-terminal acetylation, and proteolytic modification. Carbamylation by cyanate *in vitro* produced similar, if not identical, isozymes. Additional discussion of cyanate effects on enzymes and proteins was provided by Harding (92).

Oxidation/Reduction

Masters and Holmes (165) cited several cases of epigenetic enzyme variation (EST, MDH, G6PDH, CAT, CK, and carbonate dehydratase), and specifically, subbanding of LDH. Reducing agents reverted multiple bands of LDH-A_4 into one band, while incubation with glutathione resulted in the multiple pattern once again. Taketa and Watanabe (250) were able to eliminate or produce some forms of three "major" G6PDH isozymes from rat liver with various chemical treatments. The same treatments produced similar results on G6PDH from human liver and erythrocytes (276) and suggest that oxidation of sulfhydryl groups may be responsible for the heterogeneity. Reelectrophoresis of any single G6PDH band always resulted in only one staining band. Dawson and Jaeger (54) examined PGM in 18 species; PGM exhibited stable phenotypes even after months of frozen storage, however, aging of chicken liver resulted in altered patterns of PGM on starch gels. The addition of mercaptoethanol did not affect the altered patterns, while *p*-chloromercuribenzoate added to fresh samples changed the normal pattern into a pattern similar, but not identical to the aged pattern. Sulfhydryl groups of PGM were most likely involved in the modified patterns (54). Dawson and Mitchell (55) examined the interconvertibility of mammalian PGM isozymes.

Fisher and Harris (73) studied PGM isozymes in order to determine the cause(s) of additional zones of activity using starch gel electrophoresis. Seven thiol reagents were tested on the products of three PGM loci in human tissues. Phosphoglucomutase$_1$, PGM$_2$, and PGM$_3$ each exhibited nongenetic variation in at least one tissue or cell type. In placental extracts, one or two secondary isozymes often appeared after storage, which was coincident with a loss in staining intensity of the two PGM$_3$ isozymes (one primary and one secondary isozyme). Treatment of PGM$_3$ isozymes with oxidized glutathione produced a number of different phenotypes (Fig. 1). Without knowledge of the effects of the thiol reagents, Fisher and Harris (73) believed the PGM zymograms may have been interpreted incorrectly. Interestingly, the PGM phenotypes described for the guppy, *Poecilia reticulata*, by Shami and Beardmore (226) were identical to the possible phenotypes described by Fisher and Harris [(73), Fig. 1]. Shami and Beardmore (226) did not use a thiol reagent; therefore, the secondary PGM isozymes may have been oxidized at the sulfhydryl group(s). The variation in *P. reticulata* appeared to be inherited as shown by breeding data (226); therefore, it is possible that an allele encoding a PGM with a cysteine substituted for a neutral amino acid was present and remained cryptic until the cysteinyl sylfhydryl was oxidized. Hopkinson (110) described just such a phenomenon and its usefulness for detecting a genetic polymorphism in human GPI.

Conjugation, Polymerization and Dissociation

Latner and Skillen [(139), p. 2] noted that ". . . multiple bands may be produced by combination with different nonenzymatic serum proteins." Some additional LDH bands have been attributed to LDH conjugated to an immunoglobulin; immunoglobulin has been found complexed with CK, ALP and AMY as well [(29,37), references in (66, 148,169,244)]. A size variant of BChE from plasma was ac-

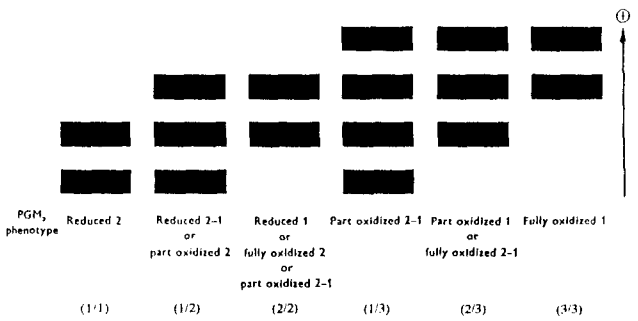

FIG. 1. Effects of different oxidation states of PGM$_3$ on formation of secondary isozymes with unique electrophoretic mobilities. Oxidation/reduction states are given below each phenotype, and the corresponding *Poecilia reticulata* phenotypes from Shami and Beardmore (1978) are given in parentheses. Reprinted from R.A. Fisher and H. Harris, "Secondary" isozymes derived from the three PGM loci. Ann. Human Genet. 36:69–77, Copyright (1972) by Cambridge University Press, reprinted with the permission of Cambridge University Press.

tually a BChE monomer bound to albumin by a disulphide bond (36). Heterogenous HB patterns have been shown to be the result of polymerization, dissociation, autoxidation and preparative method (199,204). Polymerization of HB can occur both *in vivo* and *in vitro*; polymerized HB are also referred to as heavy HB. Houston (112) detailed electrophoretic methodology for separation of fish HB and cautioned against assuming that each staining band represents a different hemoglobin due to the presence of oxy and deoxyhemoglobin and free HB vs HB-haptoglobin complexes [for example of latter see (186)].

Enzymes or proteins that are lipoproteins or membrane-bound and possibly subject to exhibiting "polymorphism" due to the lipid component (which may depend on diet or ambient temperature) include ALP, AChE, EST and succinate dehydrogenase (EC 1.3.99.1) [(59,65), in (91), (96,97, 135)].

Clausen (40) found that NADH had an effect on the mobility of human LDH isozymes; each of the 5 LDH tetramers experienced an increase in mobility that was a function of the concentration of NADH (the NADH is added to extracts, then electrophoresed, and this is when the difference in mobility is effected). Wilkinson (285) found NAD increased the mobility of LDH, and NADP altered the mobility of G6PDH (10,35). Secondary alcohol dehydrogenase "isozymes" in *D. melanogaster* were the result of bound NAD^+ or an NAD-carbonyl compound (116, 223,260). Controlling the degree of saturation of the protein with coenzyme in the extract before electrophoresis should help eliminate the effect of coenzyme binding.

Bergman *et al.* (16) discovered mobility altering of G6PDH and phosphogluconate dehydrogenase with heparin, a commonly used anticoagulant for blood collection; both enzymes from human, bovine, equine and canine blood exhibited altered mobilities due to heparin. The effect was present on starch gels, but not on agarose gels. Human phosphogluconate dehydrogenase patterns returned to normal after five days storage at 4°C (16). Heparin also interacts with cytochrome *c*, changing the thermal properties of the protein (9). Two forms of extracellular superoxide dismutase (and several other enzymes) have affinity for heparin; one superoxide dismutase has strong affinity and the other intermediate affinity [(125) and references therein, (162)]. Heparin most likely binds at the C-terminal end of superoxide dismutase which contains nine positively-charged amino acids (105). Although it has not been determined if heparin binding alters the electrophoretic mobility of this superoxide dismutase, it seems quite possible, especially if some of the positive charges are masked. Heparin effects on other enzymes and proteins should be investigated. Heparin degrades upon exposure to light (206), and this may cause even more heterogeneity. Sun (224) noted that CK activity can be inhibited by EDTA, citrate and fluoride and that CK is unstable in light. Smit *et al.* (232) compared effects of the commonly used anticoagulants, EDTA and heparin, on fish blood and concluded that heparin was preferred over EDTA due to the alteration of a number of parameters (pH and hematocrit) by EDTA. EDTA lowered the blood pH by over 1 pH unit at higher concentrations, while heparin caused only minute changes at any concentration.

Other Interconvertible "Isozymes"

Escherichia coli citrate (*si*)-synthase (EC 4.1.3.7) existed in three electrophoretically distinct isozymes, but was found to be an active tetramer in dynamic equilibrium with octameric and monomeric states as well (51,293). Reelectrophoresis of any one band resulted in the same three bands observed initially (51). Thus, the three bands of activity on gel electrophoresis resulted from migration of inactive monomers, inactive octomers and active tetramers, and following electrophoresis, some of the monomers and octomers formed active tetramers, which were visualized on the gel. Frydman *et al.* (76) studied the processes responsible for the *in vivo* interconversion of various forms of rat liver biliverdin reductase. They found three catalytically active forms of the enzyme. Molecular form 1 could be converted to form 3 by treatment of live rats with $CoCl_2$ or phenylhydrazine. Molecular form 3 could be converted back to form 1 *in vitro* with the addition of reduced thioredoxin. The above *in vivo* treatments had no detectable effect on either spleen or kidney biliverdin reductase which exists only as form 1 in these tissues. The interconversion of form 1 to form 3 involved the *de novo* synthesis of mRNA and protein since the addition of cycloheximide and actinomycin D halted the interconversion of form 1 into form 3. Form 2 was also present in minor amounts, but the genesis of this form was not determined. Frydman *et al.* (76) concluded that form 3 was an enzymatically driven posttranslational modification of form 1, possibly due to the formation of a disulfide bridge between neighboring cysteinyl sulfhydryl groups (the opposite effect produced by reduced thioredoxin). Thioredoxin is responsible for regulating enzymic activity by breaking disulfide bonds (242).

Sialic Acids and Carbohydrates

Nonenzymatic glycosylation [glycation *sensu* (252)] of HB has been demonstrated, and many sugars are known to covalently bond to HB at either the α-amino terminal residue or at the ε-amino group of lysine (92). Glycated HB can constitute up to approximately 10% of the total HB; HB AIc can occur up to 4% in normal subjects and up to 10% in diabetics (92). Normally, 20% of HB can be glycated at lysine ε-amino, but can excede 50% in diabetics. Glycation may be responsible for some HB isoforms visualized on gel electrophoresis. Harding (92) also discussed many other cases of modified HB, and many of the HB exhibited altered electrophoretic mobilities.

Sialic acid is a general term for the many known varieties of *N*-acetyl neuraminic acid derivatives (19,48), and they are often the end molecule on an already complex assemblage of sugars, but several other Sia attachment schemes are known (48,114,201). The enzyme, NEU, is used to cleave the negatively-charged Sia from carbohydrate chain attachment sites. The negative charge of Sia often alters the electrophoretic mobility of the affected protein (see cases cited below). Sialic acids are quite common in fishes, and Warren (274) gave an extensive list of fish species known to contain Sia. Corfield and Schauer (48) covered Sia distribution from bacteria to man, and more recently, Roth *et al.* (213) discovered Sia in *D. melanogaster*, including a Sia form called polysialic acid that was only present in early developmental stages. Sources of information on Sia are Schauer (218) and Rosenberg and Schengrund (211). Sialic acids are not the only carbohydrate moiety which can be attached to polypeptides (24,44,86,184,191, 240). Therefore, even if NEU successfully cleaves off Sia, there still remains a carbohydrate moiety that may influence the electrophoretic mobility due mainly to conformation/size effects. The remaining carbohydrate chains may be removed by other glycosidases, and Endo H, Endo D, and Endo F are the three most often used endo-β-N-acetylglucosaminidases for cleaving N-linked oligosaccharides from Asn residues (184). Removal of the bulk of the carbohydrate chain may induce conformational changes and/or render the protein inactive and useless for electrophoretic analysis.

The biological source of the NEU should be specified in the materials and methods section [e.g., (101,239,266)] as a future investigator may find contradictory results of NEU treatment if using NEU from a different source organism, especially since NEU from some organisms will not cleave certain Sia [e.g., *Vibrio cholerae* NEU and Neu(4,5)AC_2; (44,219)]. The methodology of incubating proteins with NEU at a high temperature (37°C) for an extended time (24 hr) (266) may introduce nongenetic variation. Most temperate zone fishes never even experience temperatures as great as 37°C. Exposure of enzymes to such a temperature would seem to predispose them to at least some degradation/denaturation. Such incubations should be carried out at a temperature close to the body temperature (≈ambient temperature) of the fish when it was captured (or temperature of acclimation). Also, the results of NEU treatment over time performed by Hershberger (101) indicate that at 37°C, after only 5 min, much of the Sia had been cleaved. Differences in the relative staining intensity of bands was evident among the various times (5,15,30,60,90, and 120 min and 12 hr); however, at the time intervals examined by Hershberger, Sia-modified TF still remained as 1–4 bands of staining activity, thus not helping to clarify interpretation concerning the number of loci. Treatment for greater than 4 hr surprisingly resulted in the same pattern as the original untreated sample as if the Sia had become reattached to the TF! Therefore, NEU treatment for 24 hr may fail to show any phenotypic change. Perhaps the NEU lost activity after approximately 4 hr.

Sialic acid-induced heterogeneity has been documented for numerous enzymes and proteins; glycoproteins known to exhibit "polymorphism" due to sialic acids or other carbohydrate groups include: AMY (70), AChE [(151,198), see Table 2)], ACP (174,233,275,292), ALP [(49,141,174, 209,235), in (236), (267,275)], aspartate aminotransferase (57), BChE (246,247), CAT (123), CAP (141), α-L-fucosidase [EC 3.2.1.5; in (214)], α-galactosidase [EC 3.2.1.22, (275)], α-glucosidase (248,292), α-mannosidase [EC 3.2.1.24, (292)], plasminogen (34), TF [(275), see Table 2], and tropinesterase [EC 3.1.1.10; (158)]. Superoxide dismutase may also be sialylated (254) and therefore, subject to electrophoretic heterogeneity. Whitmore and Goldberg (281) studied variation of *Salvelinus fontinalis* and *Salvelinus namaycush* ALP (+ additional species); they tested for attached Sia by treating samples with NEU. They found no posttranslational modifications due to Sia; however, they noted other variation which could have been due to other carbohydrate molecules. Robinson and Pierce (209) treated human serum ALP with NEU and discovered that much of the observed heterogeneity was due to Sia. Similarly, NEU treatment converted several bands of human kidney ALP to similar, if not identically migrating bands (28). Higashino *et al.* (104) also reported that some ALP heterogeneity was due to Sia attachment. NEU treatment had no effect on G6PDH from *Oncorhynchus mykiss* blood and liver (35). Heterogeneity of mouse liver CAT was due to the number of Sia attached to the tetramer (123). Pig heart cytosolic aspartate aminotransferase isozymes contain Sia and thus, heterogeneity may be due to differences in Sia content (57). 5′-Nucleotidase is a sialoglycoprotein and heterogeneity is reduced by NEU treatment [review by (298)]. Law (141) detected variation in ALP and CAP in chicken plasma that was due to Sia and suggested that another enzyme was involved that controlled sialylation/desialylation because the faster moving and more numerously sialylated forms of both enzymes always occurred together, suggesting inheritance of sialyltransferases (EC 2.4.99.6–7). Womack (292) compared published results that reported variation of ACP, α-mannosidase, and α-galactosidase due to inheritance at other loci. In all the cases, differences in sialylation of the enzymes were heritable; therefore, suggesting the involvement of polymorphic sialyltransferases or NEU. Since all the above enzymes were analyzed in the same mouse strain, Womack (292) hypothesized that a single gene may exert a pleiotropic effect on all of the enzymes through sialylation [also see (228)]. Similarly, a genetic polymorphism in a sialidase may have been responsible for mobility differences in *D. melanogaster* CAP and EST-6 (43) and in flax (*Linum usitatissimum*) peroxidase (EC 1.11.1.7), EST, and ACP (69).

Ogita (183) presented evidence that qualitative and

TABLE 2. Fish enzymes and proteins examined for the presence of sialic acids. AChE = Acetylcholinesterase, EST = Esterase, TF = Transferrin, P = present, A = absent

Species	Protein/enzyme	Sia	Number of Sia	Reference
Torpedo californica	AChE	P	?	151
Eletrophorus electricus	AChE	P	?	198
Gambusia affinis	EST	P	?	107
Clupea harengus	EST	P	?	230
Salvelinus fontinalis	TF	P	4?	101
Cyprinus carpio	TF (polymorphic)	A	—	265
Barbus meridionalis petenyi	TF (c)	P	2	237, 238
Barbus barbus	TF (B & I)	P	?	237
Tinca tinca	TF	A	—	238
Ctenopharyngodon idella	TF	A	—	238
Chondrostoma nasus	TF	A	—	238
Hypopthalmichthys molitrix	TF	P	2?	238
Aristichthys nobilis	TF	P	2?	238
Silurus glanis	TF	A	—	241
Esox lucius	TF	P	2	241
Salmo salar	TF	P	4	210

quantitative differences in human serum BChE were due to modifying factors such as NEU and/or proteolytic enzymes. Svensmark (246,247) and Harris *et al.* (95) discovered altered mobilities of human serum cholinesterases after incubation with NEU. Gasser and Rowlands (82) investigated two "variants" of human serum ChE. The "variants" were not genetically inherited as shown by family studies, and serum exhibiting normal ChE patterns and subsequently treated with NEU showed a pattern similar to the "variant" ChEs. AChE is a glycoprotein with four asparagine-linked oligosaccharides (36,151,198) and presumably can bind at least four Sia. Human "fetal-type" AChE could be converted to "adult-type" AChE with a NEU treatment preceding electrophoresis (13,80). Edwards and Shaw (65) demonstrated a shift in electrophoretic mobility for human red cell AChE treated with NEU. Differences in sialylation between identical tetramers may explain the 'cold' and 'warm' AChE isozymes found by Baldwin and Hochachka (11). Sialic acids were responsible for at least some apparent "polymorphisms" in herring, *Clupea harengus*, muscle EST (230). Additional explanations could be alternative splicing or posttranslational modification, which are responsible for some of the known secondary AChE (215,229,253). Another possible explanation for the cold and warm AChE isozymes is a difference in the glycosyl-phosphatidylinositol anchor, which attaches AChE to cell membranes and in some cases can be removed with phosphatidylinositol-specific phospholipase C (EC 3.1.4.10) [(78,79,149,150), also refer to papers in (33)]. Kominami *et al.* (135) demonstrated that the electrophoretic difference between hepatic membrane-bound ALP and serum soluble ALP was due to glycosyl-phosphatidylinositol anchors; treatment of the hepatic ALP with phosphatidylinositol-specific phospholipase C caused an increase in its electrophoretic mobility, which was identical to that of the serum ALP. Tripathi and O'Brien (256) discovered four AChE "isozymes" in head extracts of the housefly, *Musca domestica*, using polyacrylamide gel electrophoresis. Examination of the AChE "isozymes" indicated kinetic differences among them and seemed to support the notion that the "isozymes" were the products of distinct genes. However, comparisons between "normal" (susceptible) and organophosphorus-resistant strains of flies showed the four "isozymes" from the resistant strain were all insensitive to poisoning to approximately the same degree as compared to the non-resistant (normal) strain "isozymes." Tripathi and O'Brien [(256), p. 402] reasoned that: "Since it is very unlikely that four different genes would all have mutated to produce precisely the same result, we must conclude that the catalytic site of all four isozymes is under the control of a single gene, and that the variety of isozymes is epigenetic rather than genetic." The genesis of BChE "isozymes" may also explain those observed for AChE by Tripathi and O'Brien (256). Although rare, a true genetic polymorphism was discovered for human AChE (12). Esterases from *Gambusia affinis* have been shown to be affected by Sia attachment (107). They suspected the Sia played a role in stabilizing the quaternary structure of the EST. Five EST loci were present, but only EST-3 was affected by NEU. Upon treatment with NEU, 2 bands of activity were converted into 5 staining bands and 4 bands were converted into 10 bands. It was considered a result of recombination of subunits after the dimers had been destabilized by Sia removal. However, the treatment of the two forms (which were highly "saturated" with Sia) may have produced a series of progressively less "saturated" forms, resulting in the numerous EST bands (Fig. 2). Esterases from *Cepaea nemoralis* were not affected by NEU treatment (187).

Utter *et al.* (262) found EST to exhibit artifacts, and subsampling results were not consistent. Also, liver EST patterns of *O. mykiss* progeny varied from the expected based

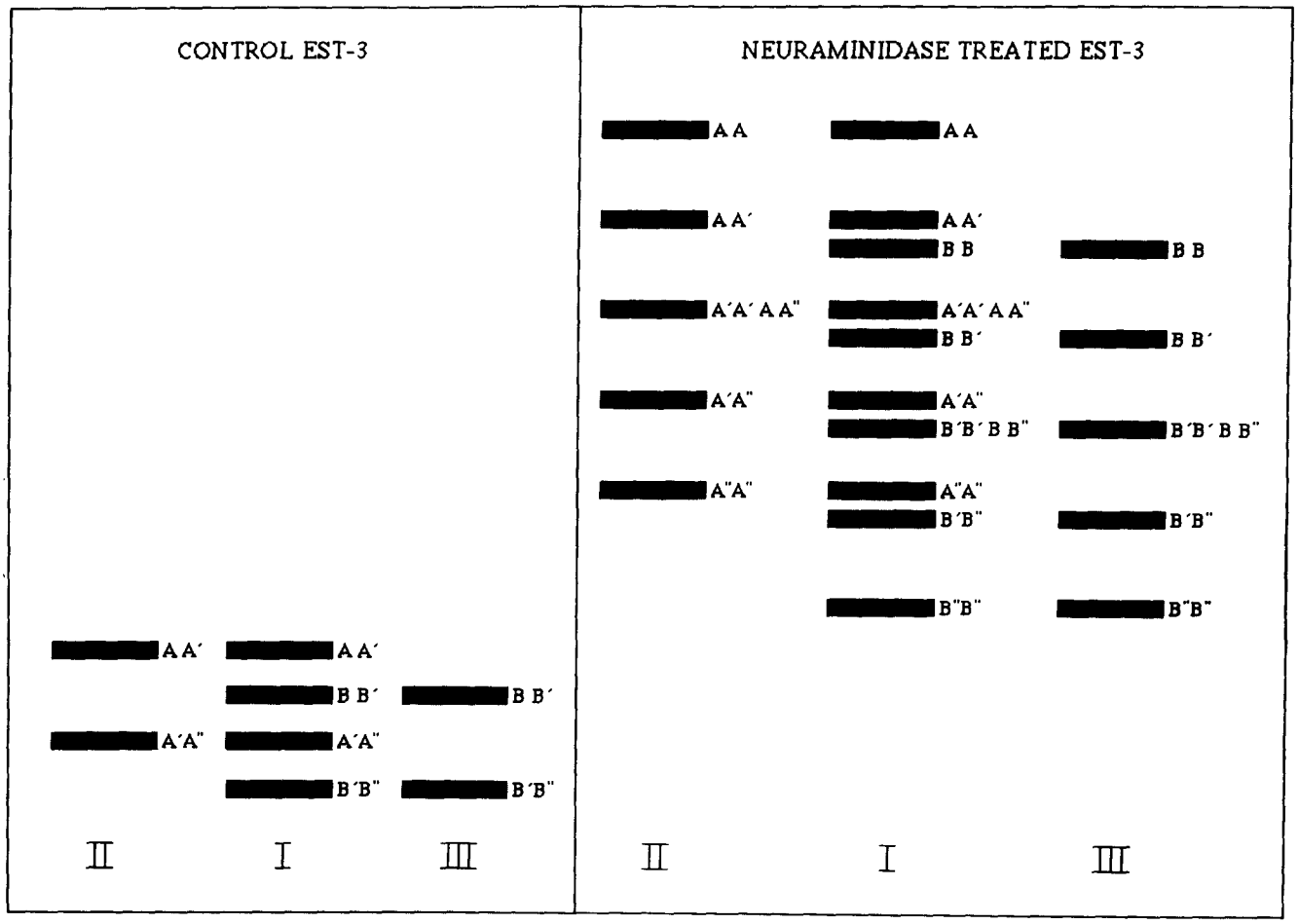

FIG. 2. Effects of exo-α-sialidase (neuraminidase) treatment on muscle EST-3 phenotypes of mosquitofish, *Gambusia affinis* (origin at top). Reprinted from Comparative Biochemistry and Physiology, 58B, D.H. Hodges and D.H. Whitmore, Muscle esterases of the mosquitofish, *Gambusia affinis*, 401–407, Copyright (1977), with kind permission from Elsevier Science Inc.

on parental phenotypes; subsampling data were consistent, however, so nongenetic effects due to storage seem to be ruled out in this case (262). Allendorf *et al.* [(3), p. 425] stated: "Inheritance studies have confirmed the genetic basis of this variation but there are indications of possible ontogenetic and environmental effects on the expression of this locus [*Est.*]" Richardson *et al.* [(206), p. 176] stated: "As a consequence of these problems the use of esterases in allozyme electrophoresis must be viewed with some caution . . . Investigative work must *always* accompany the use of any putative esterase locus as a genetic marker . . . [and] . . . the genetic basis of EST variation should be confirmed from breeding data."

TF are monomeric, nonenzymatic proteins which bind and distribute iron (as Fe^{3+}) in the body (24,75,155). Many reports exist of heterogeneity of TF, and several studies have shown that at least some of this heterogeneity is due to the attachment of Sia or other carbohydrate chains and not different primary structures (14,17,24,44,239). The number of iron atoms bound per molecule of TF can also influence electrophoretic mobility (239,297). The alteration in electrophoretic mobility may result from the conformational changes associated with binding and releasing Fe^{3+} (113), and TF is capable of binding other di- and trivalent metals as well (24), although how this would affect electrophoretic mobility has not been tested. McGovern and Tracy (152,153) discussed many published accounts covering a variety of factors that affect TF and CAP "electromorphs." On the other hand, there are studies that conclude true genetic variation does exist in the TF within/among some species of fishes (263,265). Utter *et al.* (263) did not encounter unexplained variation of TF phenotypes of rainbow trout, and their work included inheritance studies as well. However, some of the "true" genetic variation may not be within the TF themselves, but in the gene(s) which synthesize sialyltransferases which control attachment of Sia (171,234). Investigators reporting TF polymorphisms and who did not investigate these posttranslational modifica-

tions may have misinterpreted their results; some investigators did postulate that Sia may have been responsible for some of the observed variation, even when breeding studies were conducted (81). In a study of *S. fontinalis* TF, Hershberger (101) treated samples with NEU, producing a very notable effect upon the TF mobilities and phenotypic expression. Hershberger did not state whether he considered TF a dimer or monomer, but did mention that more TF bands were present than expected. Since NEU treatment never resulted in the expression of only one TF band, which would be expected for a homozygote (BB or CC) regardless of quaternary structure, it is odd that Hershberger did not find this result questionable. One probable explanation, especially when knowing TF is monomeric and brook trout are tetraploid and express some duplicate loci, is that two TF loci are expressed in brook trout, then three alleles may still be involved. Also, differences in Fe^{3+} content could have caused the banding observed by Hershberger (101). Ferguson (68) mentioned Sia as a cause of variation in pigeon (*Columba livia*) TF; the three banded pattern could be reduced to one band after treatment with NEU. Similarly, two or three TF detected in humans with starch gel electrophoresis can be converted to one band with NEU treatment (24). Chicken TF are also known to have attached Sia [(270) and references therein]. Valenta *et al.* (265) thoroughly evaluated the heterogeneity of TF in *Cyprinus carpio*; their study included inheritance data, starch gel electrophoresis, autoradiography, immunoelectrophoresis, molecular weight estimation, and NEU treatment. Apparently, the observed polymorphism of TF in carp is genetically determined, and the carp TF did not contain any Sia (265). Studies conducted to date indicate that Sia are present and exhibit effects in some fish species, while other species do not have modified TF [(266), Table 2].

Tissue-specific and Stage-specific Primary and Secondary Isozymes

Even tissue-specific expression of proteins, which along with some form of detectable structural difference (electrophoresis) is often taken as evidence that the forms are distinct gene products, may be due to alternative splicing of single gene transcripts (212) or posttranslational modification (18). Multiple mRNA products can arise from a single gene. One gene can encode two proteins that are differentially expressed between sexes [Baker in (145)], between tissues (140,159,160,179,212,216), between subcellular compartments (140,245), under different growth (media) conditions (30), and at different developmental stages (179). Rat liver cytosolic and mitochondrial fumarate hydratase (EC 4.2.1.2) are both encoded by a single gene; however, they do not differ in primary structure, and therefore, this case is distinct from others in which the multiple gene products differ in primary structure (245). An interesting example is G3PDH in *D. melanogaster*. Three "isozymes" have been found in *D. melanogaster*, and the "isozymes" exhibit at least some tissue specificity. G3PDH-3 is present in early instars, whereas G3PDH-1 and G3PDH-2 do not appear until the adult stage. All three "isozymes" are encoded by a single gene and posttranslational modification (deamidation or proteolytic modification) may have been responsible for the appearance of the two secondary G3PDH isozymes [refer to (18)].

Conformational Changes in Enzymes

The presence of "conformational isomers" of LDH and MDH has been suggested as an explanation for "extra" bands appearing on zymograms during a study of the cyprinid genus *Luxilus* (203). The LDH heterotetramer, A_2B_2, was suspected of existing in two forms in their study as well as in *Crotalus v. viridis* eye tissue (177), two species of *Rhinichthys* (41), *Pimephales promelas* (167), *Notropis stramineus* (134), and *Nocomis biguttatus* (284). Whitt *et al.* (284) also demonstrated "sub-banding" for LDH A_4 in *Lampetra appendix*. An LDH B_2C_2 isozyme formed by *in vitro* molecular hybridization was suspected of being represented by two forms (282). A conformational change was suspected for the formation of an enolase "isozyme" in *Ascaris suum* (83). "Isozyme" 3 was formed from isozyme 2 *in vitro* by a number of agents and 2-mercaptoethanol had no effect on the isozymes. Other possible causes for the enzyme conversion such as bound ligands were investigated. Although not proven conclusively, a conformational change was an explanation not ruled out (83). In this case, the two isozymes were separable by electrophoresis. Conformational changes may also result from a posttranslational modification (92). Lebherz (142) discussed several accounts of conformational isozymes and useful methods for investigating this phenomenon. Lebherz (142) also mentioned that most cases of "conformational isozymes" have provided little evidence that a conformational change has actually taken place. Other aspects of conformational changes were discussed in Poly (193).

In Vitro *Modifications*

Many of the posttranslational modifications that occur *in vivo* may also occur *in vitro*, but the cases described below resulted from *in vitro* modifications, which include changes occurring in a whole animal from storage through running of gels. Hernandez-Juviel *et al.* (100) reported significant alterations in electrophoretic mobilities of glutamate dehydrogenase (EC 1.4.1.2) from prairie rattlesnake, *Crotalus v. viridis*, as a result of varying dilutions of the enzyme extract. They found this phenomenon when using a lithium hydroxide buffer, but not when using a discontinuous borate buffer system. A number of other enzymes examined were unaffected by dilution. Hernandez-Juviel *et al.* (100) also discussed methods for detecting dilution effects on enzymes

and indicated that interpretation of minor mobility differences in glutamate dehydrogenase (and other glutamate dehydrogenases) should be made with caution. Differences in feeding or health of individuals were considered possible factors affecting *in vivo* enzyme levels, and even if all the samples were weighed and homogenized in equal volumes, the enzyme concentrations may vary enough to produce differences in electrophoretic mobility and ultimately, an incorrect assignment of nonhomology (100). Starch gel electrophoresis of conalbumin at various concentrations revealed an effect of protein concentration on the number of staining fractions (190). Gracy (89) also cited many cases of altered enzyme activity levels associated with aging. Conditions under which whole animal or protein extracts are held can influence electrophoretic results. The presence of secondary isozymes on gels has been attributed to the breakdown of proteins due to storage conditions (length of storage and insufficient temperature) (206), and particular enzymes are more susceptible to this degradation than others (261). Dessauer *et al.* (58) referred to a number of causes of protein modifications which occur during storage, including: oxidation of sulfhydryl residues, oxidation of ferrous iron, deamidation of asparagine residues, rearrangements of subunits, and conformational changes. An additional zone of ACP activity resulted from storage of blood from two species of kangaroos; the additional ACP appeared in only some of the stored samples and was not present in fresh samples from the same individuals previously shown to possess the additional ACP band (138). Similar results were shown for *Triturus cristatus carnifex* PGM between fresh and stored samples (224); the additional PGM band following storage was likely due to sulfhydryl oxidation. Storage of pure β-lactoglobulins in a Tris buffer (pH 8.7) resulted in up to three additional bands on gels after one day and up to eleven additional bands; the extra staining fractions were considered aggregations of β-lactoglobulins, possibly through disulphide bridges (2). Similar results were obtained by McKenzie and Sawyer (154) and their work supported the hypothesis of Akroyd (2) in that n-ethylmaleimide prevented the formation of any additional bands. Kobayashi *et al.* (133) found additional adenosine deaminase isozymes were the result of storage, and the additional bands complicated interpretations. The secondary isozymes were not present in fresh samples and treatment with 2-mercaptoethanol greatly reduced the artifactual bands on gels. The secondary isozymes were attributed to reactive sulfhydryl groups in adenosine deaminase (133). Many cases of secondary isozyme formation involve bands that are very light in staining intensity as compared to the original "parent" enzyme band and can give a wide range of staining intensities (178,206), including very strong bands (133), which would likely be interpreted as a distinct gene product. Dreyfus *et al.* (60) indicated that following storage ACP, adenosine deaminase, PGM, GPI, peptidase (EC 3.4.11._), x-pro dipeptidase (or peptidase D, EC 3.4.13.9), and nucleoside-triphosphate pyrophosphatase exhibited greater anodic mobility upon electrophoresis, but the mobility changes could be reduced or prevented by a reducing agent (e.g., dithiothreitol).

Quick-freezing and frozen storage of organisms is a standard method for electrophoretic studies; however, Watts (277) warned against freezing Molluscans because the hepatopancreas may be ruptured, releasing proteolytic enzymes. Upon thawing the proteolytic enzymes can rapidly degrade any enzymes they contact even at the low temperatures ($\approx$4°C) at which tissue processing and gel running are carried out (277). HB polymerization can occur *in vitro*, leading to increased heterozygosity (132). Degradation of proteins and enzymes *in vitro* due to peptidase activity has also been noted (132). Additional fructose-bisphosphate aldolase isozymes were generated due to proteolytic modifications at both C- and *N*-termini [review by (142)]. Lebherz (142) suggested that tissue homogenation and freezing/thawing released lysosomal proteases which would then act upon the enzymes. Protease inhibitors can be added to extracts in order to prevent proteolytic degradation and secondary isozyme formation (128,142). Murphy *et al.* (176) examined stability of muscle MDH, muscle myogen and liver IDHP in *Stizostedion vitreum*. The electrophoretic patterns of all three proteins were unaltered following storage at -15°C for up to 306 days or at -70°C for longer than 3 years. However, proteins differ in stability and a particular enzyme's stability may differ interspecifically, therefore, the results of Murphy *et al.* (176) should not be extrapolated to the same proteins in other species or to other proteins. The tracking dye, bromphenol blue, was responsible for heterogeneity of dihydrofolate reductase (EC 1.5.1.3) (103). Schwartz (222) treated EST allozymes with glyceraldehyde and observed gradual changes in electrophoretic mobilities of six EST allozymes (initially possessing unique mobilities) from cathodal or more cathodal positions to the same final anodic gel position. Even though the EST could be converted to identically-migrating forms, Schwartz (222) mentioned that two allozymes were present and that they differed in urea sensitivity. Several reports on the effects of ammonium persulfate on proteins appeared in 1967 (23,67,170). Mitchell (170) found multiple banding of Clostridiopeptidase B due to oxidation by ammonium persulfate, a strong oxidant used as a cross-linker in polyacrylamide gels, and observed that washing the gels to remove residual persulfate greatly reduced or eliminated artifacts.

Decompartmentation: A Cause of Secondary Isozymes?

Masters (164) discussed the subcellular localization of isozymes and suggested that displacement of isozymes from their normal compartment within the cell, which would likely happen in standard extraction procedures, could be involved in posttranslational modification and subsequent formation of secondary isozymes. Heidrich (98) determined that beef liver CAT multiplicity was due to the method of extraction employed and that one band of activity was

transformed into five when a different extraction technique was used that released the enzymes from peroxisomes into the surrounding medium. Epigenetic modifications have been suggested for CAT "isozymes" in mice as well (108). Nelson and Scandalios (180) studied CAT in the marine snail, *Nassarius obsoleta* and included several methods for the detection of interconvertibility of the CAT isozymes. They concluded that the CAT isozymes were not secondary isozymes. Rothe (214) discussed the concept of intracellular compartmentation (not decompartmentation) as a process involved in secondary isozyme formation.

Four isozymes of adenylate kinase have been found in rat liver (50); each isozyme appeared to have a fairly distinct subcellular localization as determined by differential and density gradient centrifugation and subsequent analyses. Isozyme I was found only in nuclear fractions, Isozyme II was found in cytosol, Isozyme III was predominantly in the mitochondria, although limited activity was also found in nuclei, and Isozyme IV could not be localized, although it could be consistently found in hypotonic extracts. Normal extraction procedures disrupted the subcellular distributions and released all isozymes into the cytosol (50). It is interesting that the intracellular distribution of Isozyme IV could not be determined due to very low or unstable activity (50), but that it seemed to "appear" (became detectable) after normal tissue homogenation (and decompartmentation of Isozymes I and III). If the suspicions of Masters (164) are correct, one might wonder if Isozyme IV was a secondary isozyme resulting from changes in Isozymes I or III that resulted from releasing these isozymes from their intracellular compartments. Fish (*Genypterus chilensis*) liver fructose bisphosphatase is labile to proteolytic modification during extraction, and the enzyme's catalytic properties are also significantly altered. Modification of the purification procedure eliminated the conversion of the "neutral" form to "alkaline" form (84). Johnson and Grossman (121) studied the formation of tyrosine transaminase "isozymes" utilizing a variety of extraction buffers and homogenization techniques and found that the number and quantity of the "isozymes" depended on the buffer and method of extraction. *In vivo*, apparently only one true tyrosine transaminase is present, and two secondary isozymes are often visualized by a number of separation techniques (121,122).

Some isozymes with quaternary structure may not form due to the spatial isolation of subunits (106). Also, the formation of some heteromultimers may only be facilitated in the presence (binding) of a third subunit (including subunits that are normally not bound) that confers some structural change allowing the "unusual" joining of subunits [e.g., LDH in (282)]. The formation of some isozymes *in vitro* may be the result of breaking down spatial isolation of subunits during extraction. Enzyme-containing organelles can be isolated and disrupted under conditions that avoid secondary isozyme formation (85).

Enzymes may be denatured or inactivated by poisons secreted by the organism (e.g., plants); the poisons would not be harmful *in vivo* to the source organism due to compartmentation, but destruction of the cells and vesicles *in vitro* releases the poison. The effects could result in problems with data interpretation (31,129,206).

Detection of Posttranslational Modifications

Direct sequencing of proteins to determine amino acid composition will provide valuable data in itself and also will point out possible sites were deamidation, glycosylation or phosphorylation can occur (15,273). Walsh *et al.* (271) discussed problems associated with determining covalently modified residues in conventional sequence analysis procedures in which modified residues may coelute with another amino acid in chromatographic analyses or may have the attached groups cleaved, thus reverting it back to its original base amino acid. Conventional amino acid analysis (Edman degradation) involves acid hydrolysis which destroys many posttranslationally formed residues; therefore, standard amino acid sequencing may not detect many posttranslational modifications (208,271,272,289). Wold (289) and Chin and Wold (39) discussed research aimed at the use of proteases in detecting modified residues; proteases may allow for separation of the residues without disrupting the modifications of the residue. Many modifications (both additions and deletions) are dependent upon the flanking amino acids present. Therefore, if a side chain modification is dependent on one or more of the flanking residues, the act of separation itself, regardless of how it is achieved, may cause the loss of the attached side chain molecule(s). If this can occur, identification of some posttranslational modifications would be even more difficult in some cases.

Recently, new techniques have become available for the identification of posttranslationally modified residues. Particularly useful are the techniques fast atom bombardment mass spectrometry, gas chromatography/mass spectrometry, high performance liquid chromatography and ion-exchange chromatography (4,32,38,56,249,272,290,291). Walsh *et al.* (271) suggested that multiple techniques be employed in sequence analysis to increase the likelihood of detecting modified residues. Protein structure determinations based on gene or cDNA data may be biased without considering posttranslational modification effects on the final protein product (271). The direct genetic similarities will be revealed more readily by comparison of the primary structures. Studies using cDNA may conflict with protein electrophoretic studies due to posttranslational modification, which will not be revealed by cDNA analysis and will potentially reflect differences based on electrophoretic analyses.

Usefulness of Posttranslational Modifications

Posttranslational modifications may have some value in systematic studies as do tissue-specific and ontogenetic expression (177). The value of posttranslational modification lies in those that are enzymatically catalyzed and controlled by

a modifying gene or if the posttranslational modification reveals a cryptic polymorphism [e.g., GPI in (110,173)]. Indeed, a polymorphic protease gene has been found in *E. coli*; the number and quantity of ALP "isozymes" depends upon whether the normal protease-encoding gene or a mutant gene is present (61). The protease acts on some of the ALP polypeptides by cleaving the *N*-terminal arginine residue (61,221), and the phenomenon was referred to as polymorphic posttranslational modification. The rate of acetylation of the drug, *p*-aminobenzoic acid, by an *N*-acetyltransferase was found to be dependent on the genotype of an individual for the acetyltransferase (279). Cochrane and Richmond [(43), p.182] stated that "If such posttranslational modification systems are found to be relatively common, and if in fact polymorphisms for loci controlling modification do exist in natural populations, they will provide a new sort of genetic variation which can be readily measured." Two enzymes, XDH and AO, are posttranslationally modified by at least two modifier loci in *D. melanogaster* (71). High levels of polymorphism may be due, in part, to the effects of modifier loci on the products of structural genes (71,120,292).

CONCLUSION

Electrophoresis has been and should continue to be a useful tool for taxonomy, systematics and population genetics. Additional attention to the known causes of nongenetic and genetically-controlled posttranslational modifications should improve electrophoretic data and conclusions drawn from the data. We need to eliminate the environmental and other effectors causing phenotypic changes, either *in vivo* or *in vitro*, via posttranslational modification or other processes in order to compare the true genetic relationships of the organisms under study. Elucidation of the mechanisms governing the appearance of secondary isozymes (i.e., transcriptional or posttranscriptional) would be of great interest. A few areas in which further study would be beneficial are:

1. Develop methods that control *in vitro* modification (reducing agents are used routinely).
2. Various methods of extraction should be tested to indicate how decompartmentation effects secondary isozyme formation.
3. Resolve posttranslational modifications to the enzymes commonly used in molecular systematics.
4. Increase knowledge of primary and quaternary structures for the enzymes commonly assayed (especially EST); this should include protein sequencing techniques that are sensitive in detecting posttranslational modifications.

"Thus, until further data become available, it seems prudent to regard the existence of wide-spread post-translational modification as a possibility worthy of careful consideration" [(71), p. 718].

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