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46. This last point, if true, may prove important for the construction of a general hypothesis, since receptors could be present on lymphocytes simply because they are proteins coded in the genome of all cells; the receptors could be expressed only because their presence confers no selective disadvantage to the organism: This would be the case if the lymphocyte never "saw" prostaglandins, histamine, or catecholamines in high enough concentration to affect its function. Nonrandom distribution of receptors, then, suggests that they may perform a physiologic function.
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Enzyme Polymorphism and Metabolism

Polymorphism among enzyme loci is related to metabolic function.

George B. Johnson

Since the time of Darwin and Wallace, there have been arguments concerning the evolutionary significance of patterns of natural variation. Evolutionary biologists are now involved in a controversy over the question of whether or not genetic polymorphisms at enzyme loci are maintained by selection. Sufficient experimental data now exist to indicate that they are and to suggest their role in evolutionary processes.

Polymorphism and Selection

The current controversy over the selective significance of enzyme polymorphism has roots extending back several decades in the history of population genetics to the arguments of Fisher and Wright concerning the significance of genetic drift (*1*). Now, however, the same issues are being argued in a different context. In the absence of experimental data on the amounts of genic variation being maintained in natural populations, Kimura and Crow (*2*) suggested from theoretical considerations that the maintenance of variation should entail an evolutionary cost, or "genetic load," and that because of this, the total amount of polymorphism in natural populations may not be great; excessive genetic load would be expected to result in population extinction (*3*). However, with the advent of electrophoresis as a common tool for surveying genetic variation of enzyme loci (*4*), it has become apparent that the amount of polymorphic variation at the enzyme loci of natural populations is quite high (*5–14*), far higher than could exist if the original genetic load concepts were correct. In view of these results, such concepts could be maintained most simply by assuming that no selectively important differences exist among the electrophoretic variants. Thus, the argument has been advanced (*15*) that the variant proteins contain only minor differences in tertiary structure which are sufficient to affect electrophoretic mobility but not to affect significantly the functioning of the enzyme. Because electrophoretically different alleles are seen as functionally identical, they are thought to affect the organism's fitness identically, the differences among them thus being neutral to the action of selection.

Experimental evidence is now becoming available (*16*) which permits a test of the hypothesis that enzyme polymorphisms are selectively neutral. Most reported surveys of electrophoretic variation in natural populations have provided evidence of nonrandom processes. Biogeographic patterns are often reported to be uniform over wide geographic ranges (*11, 12, 17*), or to reflect parallel patterns of environmental variation (*18–22*). Analysis of the allele frequencies reported in these studies also reveals nonrandom processes (*23*), although confusion may arise when data obtained from diverse organisms are pooled (*24*). The force of such arguments is difficult to evaluate, however, because of uncertainty about the possible involvement of migration (*25*), linkage (*26*), and founder effect (*27*). Neither is it clear when data concerning individual loci should be regarded as special cases and when they may be considered as illustrating a more general principle; those individual cases where selection is implicated at an allozyme locus do not necessarily argue powerfully for the generality of selective significance. It seems clear, however, that while some caution is necessary in evaluating the diverse array of information, much of the evidence is against Kimura's hypothesis.

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Importance of Substrate Variability

There is a more general line of argument against the hypothesis of selective neutrality, however, which proposes that degrees of enzyme polymorphism reflect physiological function, some functional classes of enzymes being far more variable than others. No combination of linkage, migration, or breeding structure can render this relationship compatible with selective neutrality.

Two hypotheses have been advanced concerning the physiological role of enzyme polymorphism. The first of these, proposed in 1968 by Gillespie and Kojima (*28*), suggests that levels of enzyme polymorphism may reflect environmental variation in substrates; the second hypothesis, which I proposed in 1971 (*29*), suggests that these polymorphisms are often associated with regulatory reactions in metabolism.

Gillespie and Kojima (*28*) pointed out that in cultures of *Drosophila ananassae*, far less heterozygosity was observed at the loci of enzymes involved in energy production than at other enzyme loci (*30*). More detailed examination of natural populations by Kojima *et al.* (*8*) bore out their original observation (*28*) and these workers suggested that the greater variability in "nonglucose metabolizing" enzymes might reflect greater variability in their substrates, as many of these substrates originate in the external environment.

Assignment of environmental origin to the substrates of enzymes may be ambiguous when the functional roles of enzymes encompass both groups. Alcohol dehydrogenase and octanol dehydrogenase may both function in lipid metabolism, as well as possibly playing a role in the breakdown of dietary alcohols (*31–36*); most carboxyesterases probably function in *Drosophila* to break down secondary compounds of plants, but some esterases undoubtedly have more restricted internal metabolic roles. Kojima and his co-workers (*8*, *28*) assigned loci whose enzymes utilize both classes of substrate to the "external substrate" class. It is important to realize, however, that in these cases the classes are not mutually exclusive.

In the several years since this dichotomy of enzyme types was proposed, there have been numerous electrophoretic investigations of enzymes, and the number of flies that have been collected from natural populations and examined in this way now exceeds 30,000. Extensive work has also been conducted on small vertebrates and on man. Upon analysis, these data strongly support the original hypothesis of Gillespie and Kojima (*28*). Among 13 *Drosophila* species, the enzymes of broad specificity, many of which utilize substrates originating from the external environment, are far more variable than those enzymes which utilize specific metabolically produced substrates (*37*).

Thus, polymorphic variation at enzyme loci correlates with the physiological variability with which the enzymes must interact in functioning. Although the groupings discussed above are broad and not always clearly defined, the general result seems valid for a wide variety of data.

Regulatory Enzymes and Metabolic Organization

In describing my hypothesis on the physiological role of enzyme polymorphism, I suggested that "those enzymes which exert acute control over flow through (metabolic) pathways should be most individually sensitive as sites of action of selective forces" (*29*). The rationale of this argument is that selection must act ultimately upon the reproductive fitness of individuals. The contributions of particular metabolic sequences to that fitness must be considered in terms of overall pathway output rather than in terms of specific reactions. Changes at loci whose enzymes regulate flow through pathways would be expected to produce far greater alterations in fitness than changes affecting enzymes which do not regulate metabolic flow.

Krebs was the first to point out that reversible reactions cannot produce major metabolic changes; only at essentially irreversible reactions may control be exercised over the rate and direction of metabolic processes (*38*). Some reactions are so rapid that near-equilibrium proportions always obtain between substrates and products of the reactions; other reactions, because of low activity or for other reasons, do not establish equilibrium. Because the nonequilibrium enzymes are potentially rate-limiting, they present opportunities for metabolic regulation of flux through the pathway. Equilibrium enzymes, on the other hand, have potential activities far in excess of the normal flux rate; modulation of the flux by regulation of these enzymes is impracticable, because activities would have to be reduced by several orders of magnitude. The role of chemical equilibria in the regulation of metabolism has been discussed by numerous workers (*39–41*).

To estimate the regulatory character of polymorphic enzymes, I suggested that the equilibrium constant (K_{eq}) of the reaction being catalyzed could be used, reasoning that thermodynamically irreversible reactions tend to be sites of metabolic regulation. This approach, although it yielded suggestive results with the limited amount of data then available, has not proved to be adequate, because many thermodynamically reversible reactions are also sites of regulation. It has been criticized by several workers (*35, 42*). K_{eq} is at best a poor indicator of regulatory involvement, because other factors may be equally important in regulation: The activity of the initial reaction of an anabolic pathway is often tightly controlled (*43–45*); similarly, branch-points in metabolism are almost always regulated, as are multifunctional enzymes (*46*, *47*). The large array of enzyme loci that have been examined for polymorphism within the last 2 years includes many whose regulatory roles have been well characterized, permitting a more detailed assessment of my hypothesis than was possible previously.

The metabolic role of specific enzymes. Almost all metabolic processes are regulated to some degree. The widespread occurrence of feedback regulation of biosynthetic pathways is well documented. At least as important is the control of thermodynamically "irreversible" reactions which must operate in both directions. Such control is achieved by employing different reaction mechanisms under separate regulatory control; for example, phosphorylase and glycogen synthetase catalyze the same reaction in opposing directions by very different mechanisms and are subject to different controls. Such dual reactions provide much of the flexibility of response characteristic of intermediary metabolism.

Krebs and Kornberg (*48*) have suggested that it is often possible to predict which enzymes in a pathway are of regulatory importance from knowledge of the sequence of the reactions. Targets of allosteric regulation would be (i) enzymes catalyzing initial reactions in pathways, (ii) enzymes catalyzing reactions immediately after a branch in a pathway, and (iii) enzymes catalyzing "futile cycles" (two separate

Table 1. The metabolic role of enzymes examined for polymorphism: P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; PRPP, phosphoribosyl pyrophosphate.

Enzyme	E.C. No.	Reaction	Physiological function
N-acetylhexosaminidase	3.2.1.30	Hydrolyzes odd-numbered oligosaccharides to yield *N*-acetylglucoseamines	Breakdown of starch
Adenine phosphoribosyl transferase	2.4.2.7	Adenine + PRPP $\rightleftharpoons$ AMP + PP_i	Purine salvage (*64*)
Adenosine deaminase	3.5.4.4	Adenosine + $H_2O \rightleftharpoons$ inosine + NH_3	First step in adenosine purine salvage pathway
Adenylate kinase	2.7.4.3	ATP + AMP $\rightleftharpoons$ 2 ADP	Modulate ATP/ADP ratio (*44, 65*) thus affecting metabolism generally
Alcohol dehydrogenases	1.1.1.1	Acetaldehyde + NADH $\rightleftharpoons$ ethanol + NAD^+, or glyceraldehyde + NADH $\rightleftharpoons$ glycerol + NAD^+	Physiological role of reverse action is not clear (*34*), a different "microsomal" system (not ADH) appears to be responsible for ethanol oxidation in mammalian liver (*33*); vitamin A synthesis (*66*); controls branchpoint in fructose and fat metabolism (*34, 67*)
Aldehyde oxidase	1.2.1.3	Acetaldehyde + $NAD^+ \rightleftharpoons$ acetate + NADH	Regulates vitamin B_6 concentration (*35*); regulates amino acid and glycogen metabolism
Aldolase	4.1.2.13	Fructose diP $\rightleftharpoons$ glyceraldehyde-3-P + DHAP	Glycolysis
Amylase	3.2.1.1	Hydrolysis of α-1,4-polyglucosans	Breakdown starch and glycogen (*68*)
Carbonic anhydrase	4.2.1.1	$CO_2 + H_2O \rightleftharpoons H_2CO_3$	Governs blood *p*H and $[CO_2]$
Catalase	1.11.1.6	$H_2O_2 \rightleftharpoons H_2O + \frac{1}{2}O_2$	Removal of hydrogen peroxide (*69*)
2,3-Diphosphoglycerate mutase	2.7.5.4	1,3-DPG $\rightleftharpoons$ 2,3-DPG	2,3-DPG modulates O_2 loading of hemoglobin (*44, 70*)
Enolase	4.2.1.11	2-Phosphoglycerate $\rightleftharpoons$ phosphoenolpyruvate + H_2O	Glycolysis
Esterases		Hydrolysis of ester linkages	Various; primarily breakdown of ingested esters (*71*)
Fumarase	4.2.1.2	Fumarate + $H_2O \rightleftharpoons$ malate	Tricarboxylic acid cycle
Galactose-1-P uridyl transferase	2.7.7.e	Galactose-1-phosphate $\rightleftharpoons$ UDP galactose	Rate-limiting step in galactose metabolism
Glucose-6-P dehydrogenase	1.1.1.49	Glucose-6-phosphate $\rightleftharpoons$ gluconic-6-phosphate	Modulates entry of hexoses into pentose metabolism
Glutamate-oxaloacetate aminotransferase	2.6.1.1	Oxaloacetate + glutamate $\rightleftharpoons$ aspartate + α-ketoglutarate	Cytoplasmic transamination (*72, 73*); mitochondrial glutamate metabolism
Glutamate-pyruvate aminotransferase	2.6.1.2	Pyruvate + glutamate $\rightleftharpoons$ alanine + α-ketoglutarate	Alanine biosynthesis; final acceptor of NH_3 in cytoplasmic transamination (*72, 73*)
Glyceraldehyde-3-P dehydrogenase	1.2.1.12	Glyceraldehyde-3-P $\rightleftharpoons$ phosphoglycerate	Glycolysis; serine biosynthesis
α-Glycerophosphate dehydrogenase	1.1.1.8	Dihydroxyacetone P + NADH $\rightleftharpoons$ α-glycerophosphate + NAD^+	In insects: regeneration of NAD^+ (*74*); in vertebrates: links glycolysis and fat synthesis (*75*)
Glyoxalase II	3.1.2.6	Hydroxyacylglutathione + $H_2O \rightleftharpoons$ glutathione + lactate	
Guanase	3.5.4.3	Guanine + $H_2O \rightleftharpoons$ xanthine + NH_3	Purine salvage (*64*)
Hexokinase	2.7.1.1	Glucose + ATP $\rightleftharpoons$ glucose-6-P + ADP	Glucose mobilization
Hypoxanthine phosphoriboxyl transferase	2.4.2.8	Guanine + PRP $\rightleftharpoons$ GMP + PP_i	Purine salvage (*64*)
Isocitrate dehydrogenase*	1.1.1.42	Isocitrate + NADP $\rightleftharpoons$ αKG + CO_2 + NADPH	? (*75*)
Isocitrate dehydrogenase†	1.1.1.41	Isocitrate + NAD $\rightleftharpoons$ αKG + CO_2 + NADH	Rate-limiting step in tricarboxylic acid cycle
Inosine triphosphatase		Inosine triphosphate $\rightleftharpoons$ inosine + PP_i + P_i	Purine catabolism
Lactate dehydrogenase	1.1.1.28	Pyruvate + NADH $\rightleftharpoons$ lactate + NAD^+	Vertebrates: control of NAD^+ (*76*)
Malate dehydrogenase*	1.1.1.37	Malate + $NAD^+ \rightleftharpoons$ oxaloacetate + NADH	NAD^+/NADH shuttle (*40, 45*)
Malate dehydrogenase†	1.1.1.37	Malate + $NAD^+ \rightleftharpoons$ oxaloacetate + NADH	Tricarboxylic acid cycle (*77*)
Malic enzyme*	1.1.1.40	Malate + $NADP^+ \rightleftharpoons$ pyruvate + CO_2 + NADPH	Produces NADP which limits rates of synthetic reactions (*78*)
Malic enzyme†	1.1.1.40	Malate + $NADP^+ \rightleftharpoons$ pyruvate + CO_2 + NADPH	Produces NADP which limits rate of adrenal steroid synthesis (*79*)
Nucleoside phosphorylase	2.4.2.1	Inosine + $P_i \rightleftharpoons$ hypoxanthine + ribose-1-P	Purine salvage (*64*)
Pepsinogen	3.4.1.1	Pepsin acts to hydrolyze peptides	Protein catabolism (*80*)
Peptidase		Protein hydrolysis	Protein catabolism (*80*)
Phosphoglucoisomerase	5.3.1.9	Glucose-6-P $\rightleftharpoons$ fructose-6-P	Glycolysis
Phosphoglucomutase	2.7.5.1	Glucose-6-P $\rightleftharpoons$ glucose-1-P	Gluconeogenesis
6-Phosphogluconate dehydrogenase	1.1.1.43	6-Phosphogluconate $\rightleftharpoons$ ribulose-5-P + CO_2	Pentose shunt
Phosphoglycerate kinase	2.7.2.3	3-Phosphoglycerate + ATP $\rightleftharpoons$ 1,3-diphosphoglycerate + ADP	Glycolysis
Phosphatase	3.1.3.1,2	R-O-P + $H_2O \rightleftharpoons$ R-OH + P_i	Various (*81*)
Phosphofructokinase	2.7.1.11	Fructose 6-P + ATP $\rightleftharpoons$ fructose-1,6-diP + ADP	Rate-limiting step in glycolysis
Pyrophosphatase	3.6.1.1	PP $\rightleftharpoons$ 2 P_i	Renders PP_i-producing reactions irreversible (*82*)
Pyruvate kinase	2.7.1.40	Pyruvate + ATP $\rightleftharpoons$ phosphoenolpyruvate + ADP	Glycolysis
Sorbitol dehydrogenase	1.1.1.14	Sorbitol + $NAD^+ \rightleftharpoons$ fructose + NADH	
Triose phosphate isomerase	5.3.1.1	3-Glyceraldehyde-P $\rightleftharpoons$ dihydroxyacetone-P	Glycolysis
Xanthine dehydrogenase	1.2.3.2	Hypoxanthine + $NAD^+ \rightleftharpoons$ xanthine + NADH; xanthine + $NAD^+ \rightleftharpoons$ urate + NADH	Purine salvage (*83*); pigment metabolism in insects (*84*)

* Cytoplasmic. † Mitochondrial.

reactions that together comprise a forward and a reverse reaction at one step in a pathway). Enzymes requiring a common specific nucleotide cofactor might act to modulate each other's level of activity. This generalization is a valid one (*49*). The role of such intermediary metabolic enzymes in regulation has been well characterized in several detailed reviews (*43–45*).

Experimental verification of postulated regulatory involvement is not, however, as simple and straightforward as the foregoing discussion might suggest. Many factors complicate regulatory patterns: intracellular compartmentalization of metabolites, metabolic differences between tissues, developmental alterations in metabolism, species differences, responses to divergent physiological conditions. Simple statements concerning modes of biochemical regulation can be made only for a given pathway operating in a particular tissue of a particular species under specified physiological conditions. Even then, the amount of regulatory influence may be relative, some amount of diffuse control being exercised by all steps of the pathway (*50*).

It is possible to make limited generalizations, however: (i) While all reactions may affect flux through a pathway to some degree, primary control is generally exercised at one or a few key reactions. (ii) When regulation of a pathway differs for different tissues or physiological states, the rate-limiting steps are usually found to be restricted to a small group of reactions. For most metabolic sequences, it is possible to characterize a limited number of reactions which are potential sites of primary metabolic control.

To identify these potential regulatory reactions, one must study their activity in vitro and in vivo. Often, one must first determine "enzyme capacity" (rate under optimal conditions of temperature, *p*H, saturating substrate, for example), using purified preparations.

Table 2. The regulatory role of enzymes examined for polymorphism. The regulatory roles of 19 enzymes in Table 1 that are not shown here have not been widely studied; all but five of them have catabolic functions. I have categorized all but 2 of the 19 enzymes not shown here as nonregulatory; the exceptions, alcohol dehydrogenases and glutamate-pyruvate aminotransferase, occupy metabolic positions of potential regulatory importance.

Regulatory enzymes		Nonregulatory enzymes	
Name	Evidence	Name	Evidence
Adenosine deaminase	Sigmoidal kinetics in vitro (*40, 47, 85*); radioactive tracer analysis of participation in in vivo pathway (*86*)	Aldolase	Substrate/product ratio at equilibrium; isotope randomization indicates reaction is very fast (*41, 44, 45, 70, 87, 88*)
Adenylate kinase	Substrate/product ratio significantly displaced from equilibrium (*65*)	Carbonic anhydrase	Reaction kinetics very fast (*89*)
Aldehyde oxidase	Regulates vitamin B_6 concentration and thus affects amino acid and glycogen metabolism (*36*)	Enolase	Substrate/product ratio at equilibrium (*87, 90*)
2,3-Diphosphoglycerate mutase	Lowest enzyme capacity relative to other steps in pathway, with metabolite interactions taken into account (*70*)	Fumarase	Substrate/product ratio at equilibrium (*90, 91*)
Galactose-1-P uridyl transferase	Pulse label experiments suggest this reaction is rate limiting (*92*)	α-Glycerophosphate dehydrogenase (insect)	Operates in DHAP-α-GP shuttle; substrate/product ratio not affected by glycolytic flux upon flight initiation (*74*)
Glucose-6-P dehydrogenase	Sigmoidal kinetics; sensitivity of turnover rate to physiologically significant factors; substrate/product ratio significantly displaced from equilibrium (*93*)	Isocitrate dehydrogenase*	Substrate/product ratio at equilibrium (*75, 87*)
Glyceraldehyde-3-P dehydrogenase	Substrate/product ratio significantly displaced from equilibrium (*45, 70, 94*)	Lactate dehydrogenase	Operates in NAD/NADH couple; substrate/product ratio at equilibrium (*41, 44, 45, 70, 76, 95*)
α-Glycerophosphate dehydrogenase (mammalian)	Allosteric kinetics (*96*); analysis of glycolytic intermediates in substrate-depleted cells indicates: (i) DHAP-α-GP shuttle is not operative; (ii) temporal patterns of α-GPDH activity (*75, 97, 98*)	Malate dehydrogenase*	Substrate/product ratio at equilibrium (*77*)
Hexokinase	Substrate/product ratio significantly displaced from equilibrium (*41, 44, 45, 70, 99*)	Malate dehydrogenase†	Substrate/product ratio at equilibrium (*87*)
Isocitrate dehydrogenase†	Sigmoidal kinetics; substrate/product ratio may be significantly displaced from equilibrium (*41, 72*)	6-P-Gluconate dehydrogenase	Substrate/product ratio at equilibrium (*93, 95*)
Malic enzyme*	Activity responsive to dietary conditions; limits rate of fat synthesis in adipose tissue (*78*)	Triose phosphate isomerase	Substrate/product ratio at equilibrium (*70, 87*)
Malic enzyme†	"Kinetically irreversible"; limits rate of adrenal steroid synthesis (*79*)		
Phosphoglucose isomerase	Substrate/product ratio sometimes displaced from equilibrium (*100*)		
Phosphoglucose mutase	Substrate-product ratio significantly displaced from equilibrium (*101*)		
Phosphoglycerate kinase	Substrate/product ratio significantly displaced from equilibrium (*87*)		
Phosphofructokinase	Substrate/product ratio significantly displaced from equilibrium (*40, 41, 44, 45, 97, 102*)		
Pyruvate kinase	Substrate/product ratio significantly displaced from equilibrium (*40, 41, 44, 45, 103*)		
Xanthine dehydrogenase	Enzyme activity responds to nutritional condition (*104*)		

* Cytoplasmic. † Mitochondrial.

However, while the enzyme observed to have the lowest capacity in vitro is potentially rate-limiting, further work is required to establish that this is true in vivo.

By studying the interactions of purified enzymes with other cellular components, it is often possible to demonstrate allosteric interactions and feedback control relationships. It is important to remember, however, that metabolic systems function as integrated units. If one step of a sequence is found to be rate-limiting, other steps may not simultaneously regulate the flux through the pathway: hence, the demonstration in vitro of allosteric inhibition of an enzyme by a pathway metabolite does not imply that regulation is achieved by this means in vivo. Clearly, metabolic regulation must be examined directly in vivo.

When a metabolic sequence is operating at steady state in vivo, each reaction proceeds at an identical rate. Thus, under steady-state conditions most enzymes of a pathway are working well below their optimal capacity, while one or a few enzymes dictate the rate of flux through the pathway. To identify the steps regulating flow, one may vary the flux through the pathway, monitoring the concentrations of intermediates. The ratios of substrate to product for all uncontrolled reactions are independent of the rate of overall flux through the pathway. Rate-limiting or controlled reactions, however, show a deviation from the equilibrium ratio of product to substrate, and the deviation varies with rate of flux through the pathway. Hence, when the observed ratio of substrate to product deviates significantly in vivo from the ratio predicted by its thermodynamic equilibrium, this can be taken as prima facie evidence that the reaction rate is being regulated or is of regulatory importance in the overall pathway.

Estimates of metabolite concentrations obtained from perturbed pathways in vivo are not certain indicators of regulatory involvement, because intracellular compartmentalization may produce misleading results. Such estimates do provide, however, the best general characterization of metabolic regulatory involvement currently available. A wide variety of enzymes in many organisms and tissue systems have been studied in this way.

Polymorphism has been investigated in all the enzymes shown in Table 1. A wide variety of synthetic and catabolic reactions are represented. Table 2 lists those reactions which have been examined as potential sites of metabolic control. In most instances, enzymes were assigned to categories according to the values reported for substrate/product ratios in vivo, relative to expected equilibrium values. When the reported substrate/product ratios did *not* deviate significantly (by greater than one order of magnitude) from equilibrium, the reaction was characterized as "nonregulatory."

Patterns of polymorphism. Tables 3 to 5 present a compilation of available data on the degrees of enzyme polymorphism observed at the loci of the enzymes listed in Table 1. That the degree of heterozygosity at an enzyme locus reflects the regulatory role of the

Table 3. Enzyme polymorphism in *Drosophila*. Because between-species homologies are often uncertain for variable-substrate loci such as esterases, mean heterozygosity is determined for all loci examined within each "variable substrate" reaction type; the reaction type is then treated as a single locus in determining mean species heterozygosity. This treatment also tends to minimize differences observed between species due to differing proportions of esterases, for example, included in the surveys. $\overline{HET}$: mean heterozygosity. Mean species heterozygosity is calculated as the average of the values of Table 3. The following *Drosophila* species are included in the analysis: aff, *D. affinis* (*8*); ath, *D. athabasca* (*8*); bip, *D. bipectinata* (*10*); equi, *D. equinoxialis* (*5*); mal, *D. malerkotliana* (*10*); mel, *D. melanogaster* (*8*); obs, *D. obscura* (*11*); paul, *D. paulistorum* (*9*); para, *D. parabipectinata* (*10*); pseu, *D. pseudoobscura* (*7*); sim, *D. simulans* (*8*); sub, *D. subobscura* (*12*); will, *D. willistoni* (*6*).

Locus	*Drosophila* species													
	aff	ath	bip	equi	mal	mel	obs	paul	para	pseu	sim	sub	will	$\overline{HET}$
Variable substrate														
Acid phosphatases	0.21		0.67	0.30	0.69	0.04		0.12	0.08	0	0.41		0.14	0.27
Alkaline phosphatases	0.35	0	0.08	0.15	0.01	0	0.16		0			0.07	0.26	0.11
Peptidases			0.04	0.45	0.01		0.03	0.27	0	0.16		0.40	0.64	0.22
Esterases	0.71	0.70	0.39	0.30	0.56	0.27	0.20	0.32	0.38	0.76	0.66	0.07	0.26	0.43
Octanol dehydrogenases	0.74	0.11	0.21	0.15	0.13	0.20	0	0.11	0	0.06	0.19		0.10	0.17
Overall														0.24
Regulatory enzymes														
Adenylate kinase				0.47*									0.25*	0.34
Alcohol dehydrogenase	0.43	0.11	0.44	0.23	0.39	0.48	0.34	0.01	0.57		0.24	0.01	0.11	0.28
Aldehyde oxidase	0.45	0.27	0.68		0.68	0.16	0.07		0.19	0.06	0.33	0.11		0.30
Glyceraldehyde-3-P dehydrogenase				0.20									0.15	0.18
Glucose-6-P dehydrogenase			0.33		0.31	0.26	0		0	0.01	0	0		0.11
Hexokinase			0.05	0.15*	0	0.10			0				0.05*	0.08
Malic enzyme	0	0.13	0.02	0.31*	0.04	0	0.09	0.06	0		0	0.15	0.09*	0.08
Phosphoglucose isomerase			0.40		0.37	0			0.46		0.01			0.25
Phosphoglucomutase		0.09	0.19	0.44	0.11	0.32		0.05	0.35		0.06		0.18	0.20
Xanthine dehydrogenase		0.02	0		0	0	0.22		0	0.56	0.05	0.29		0.13
Overall														0.19
Nonregulatory enzymes														
Aldolase	0	0.02	0	0.30	0	0			0		0.03		0.14	0.05
α-Glycerophosphate dehydrogenase	0	0	0	0.03	0.02	0.25		0.01	0.04		0.03	0	0.03	0.04
Isocitrate dehydrogenase†		0	0.49	0.09	0.49	0	0.06		0		0.20	0	0.06	0.14
Fumarase	0.06	0	0.05		0.02	0.03			0.11		0			0.04
Malate dehydrogenase	0	0	0.03	0.01	0.02	0.02	0	0.01	0	0.08	0.01	0.04	0.06	0.02
6-Phosphogluconate dehydrogenase			0.08		0.05	0.03			0		0			0.06
Triose phosphate isomerase				0.04				0.03			0		0.04	0.04
Overall														0.06
$\overline{HET}$	0.27	0.11	0.22	0.20	0.20	0.14	0.11	0.10	0.11	0.21	0.14	0.10	0.18	

* Values of $\overline{HET}$ include additional loci of this enzyme. † Cytoplasmic.

enzyme seems to be a principle of broad validity. For *Drosophila*, the probability that polymorphism at regulatory and at nonregulatory loci is not significantly different is very small, $P < .001$ (*37*). For small vertebrates, the probability is $P < .05$ (*37*), and for man it is $P \ll .001$ (*37*). It thus seems that the physiological role of polymorphism occurring at the loci of metabolic enzymes is related to metabolic regulation.

Even though an ecologically varied collection of *Drosophila* species is considered in Table 3, the magnitude of the variance within the genus is surprising. Biogeographic patterns of enzyme polymorphism may reflect environmental factors (*18–22*); indeed, I have observed clines in polymorphism within a single population of Colorado *Colias* butterflies along an alpine-montane transect (*51*). If within-species variability is strongly influenced by environmental factors, it is perhaps not unreasonable that a collection of species from very diverse localities, each sampled but a few times, should exhibit variability in degrees of polymorphism. Examination of a large number of species, such as is presented in Table 3, should minimize the effects of this variability upon comparisons of polymorphism at loci under environmentally mediated selection.

Variation in available substrate concentrations may also affect metabolic rates (*38*). While this generalized form of metabolic modulation does not appear to be highly correlated with polymorphism, it does present a possible alternative target for polymorphism in individual cases. Thus, much of intermediary metabolism is coordinated by relative concentrations of nucleotide cofactors, which are in turn affected, at least potentially, by a wide variety of reactions utilizing these cofactors. Polymorphism of vertebrate lactate dehydrogenase (LDH) and insect α-glycerophosphate dehydrogenase (α-GPDH) may reflect such indirect control. The data in Tables 3 to 5 indicate, however, that most enzyme polymorphism is associated with allosteric enzymes which are themselves points of metabolic regulation.

The role of temperature. Variation in the nature or concentration of a substrate utilized by a particular enzyme, or in its activity, usually has effects which are limited to one or a few pathways. Exceptions to this generalization are reactions involving the nucleotide cofactors. These compounds participate in reactions throughout metabolism:

Table 4. Enzyme polymorphism in small vertebrates. Included in the analysis of Selander and Johnson (*105*) are 12 rodent species, a passerine bird, a fish, and eight species of lizards. Listed values of mean heterozygosity ($\overline{HET}$) are those for polymorphic species. When all species are considered, rather than restricting the analysis to species observed to be polymorphic, overall values of average heterozygosity are reduced by about 50 percent.

Locus	$\overline{HET}$
Variable substrate	
Esterases	0.30
Indophenol oxidase	0.20
Peptidase (*106*)	0.17
Overall	0.22
Regulatory enzymes	
Alcohol dehydrogenase	0.21
Isocitrate dehydrogenase*	0.01
Glucose-6-P dehydrogenase	0.03
α-Glycerophosphate dehydrogenase	0.10
Malic enzyme	0.20
Phosphoglucose isomerase	0.16
Phosphoglucomutase 1	0.14
Phosphoglucomutase 2,3	0.24
Overall	0.14
Nonregulatory enzymes	
Glutamate-oxaloacetate aminotransferase†	0.08
Glutamate-oxaloacetate aminotransferase*	0.01
Isocitrate dehydrogenase†	0.13
Lactate dehydrogenase 1	0.09
Lactate dehydrogenase 2	0.03
Malate dehydrogenate†	0.04
Malate dehydrogenate*	0.07
6-Phosphogluconate dehydrogenase	0.11
Sorbitol dehydrogenase	0
Overall	0.06

* Mitochondrial. † Cytoplasmic.

concentrations of nicotinamide adenine dinucleotide (NAD^+) determine the operational redox potential of many respiratory reactions; similarly, concentrations of nicotinamide adenine dinucleotide phosphate (NAD^+) govern many synthetic reactions; the concentrations of ATP, ADP, and AMP (adenosine tri-, di-, and monophosphate, respectively) are universally important both as effectors and stoichiometrically. Reactions that govern the concentrations of available nucleotide cofactors thus provide a means whereby generalized metabolic control might be exercised.

Fluctuating temperature may encourage polymorphic variation at such loci. A temperature change alters all metabolic rates, thus slowing or quickening metabolism generally (*52*). Polymorphic variation at the loci of enzymes governing the ratios of ATP/AMP, $NAD^+/NADH$, or $NADP^+$/NADPH [enzymes such as adenylate kinase (AK), LDH in mammals, α-GPDH in insects, and malic enzyme (ME)] would make possible a generalized response to such generalized metabolic fluctuation. What little data exist are consistent with such a hypothesis. In homeotherms such as man, in which metabolic temperature is uniform, LDH is not observed to be significantly polymorphic. In poikilotherms such as fish, LDH is often polymorphic, and the variation seems to be related to environmental temperature (*22*): clines in allele frequency are reported which reflect latitudinal clines in water temperature. In insects, α-GPDH seems to be more polymorphic among *Drosophila* populations of temperate regions than among tropical populations. Among the 18 reported studies of tropical populations, the mean heterozygosity of α-GPDH was 0.02 and no population exceeded 0.10; among 12 studies of temperate regions, the mean heterozygosity was 0.22 and eight populations exceeded 0.10. In the temperate eastern United States, a cline in α-GPDH heterozygosity is reported which is positively correlated with latitude (*20*). The enzymes AK and ME are often polymorphic in both homeotherms and poikilotherms; because they may also play important regulatory roles, it is not possible to discern any involvement of temperature without additional data.

The latitudinal clines of polymorphic gene frequency observed in poikilotherms suggest that temperature may play an important role in selection for these polymorphisms, although thermal effects upon kinetic behavior have been demonstrated only for LDH (*22*). As no major overall difference is seen in the degree of heterozygosity between polymorphic loci of homeotherms and those of poikilotherms, a regulatory hypothesis would argue against temperature playing an exclusive role in maintaining all these polymorphisms, although polymorphic loci do occur more frequently in poikilotherms.

Isozymes

It is now well documented that isozymes (alternative subunit assemblies of multimeric enzymes coded for by more than one locus) may have differing kinetic properties (*52*). Relative concentrations of isozymes often differ from one tissue to another and may change during the course of development (*53*). Perhaps variations in isozyme distributions also reflect a mechanism of metabolic modulation. Differentiation and developmental processes in insects and vertebrates are highly integrated and buffered against

random change; the pattern of their metabolic regulation should be similar in all individuals. For such a regulatory role, isozymic modulation, identical for all individuals, is a reasonable strategy. By contrast, intermediary metabolism may experience far greater random variability; this is particularly true of pathways related to diet and respiration in adults. No one metabolic strategy would be able to cope with all situations. Enzyme polymorphism may reflect a response to such variable metabolic input. The possible involvement of polymorphism and environmental unpredictability has been widely discussed (*54*).

Although it seems unlikely that isozymes and enzyme polymorphisms reflect identical modes of regulation, the evolutionary strategies are clearly related. On theoretical grounds, one would expect tandem duplications (from which isozymes might originate) to become established in populations most readily at polymorphic loci (*55*). This suggests that tissue-specific differences in isozymes may occur more frequently at polymorphic loci. If, as I have shown, enzyme polymorphism is most frequent at regulatory enzyme loci, then one might expect tissue-specific isozymes to occur most frequently at such loci also. Values reported in the literature support this conjecture (*56*). It is more difficult to ascribe a simple evolutionary role to isozymes which occur in multiple forms within a single tissue type. Many such isozymes catalyze nonregulatory reactions. Perhaps, as has been suggested for aldolase (*53*), the different forms have different membrane-binding behavior and function in intracellular compartmentalization.

Summary and Conclusions

Polymorphism has been observed at a variety of enzyme loci in many species of insects and other animals. The high degrees of polymorphic variability that occur might be attributed to any of a variety of physiological interactions. Superior structural stability and differential substrate binding of heterologous (heterozygote) enzymes are logical avenues of investigation. Consideration of the metabolic roles of these enzymes suggests another alternative, that enzyme polymorphisms may have a metabolic regulatory function. The relationships observed suggest that enzyme polymorphisms increase fitness by providing a means of metabolically compensating for a varying environment. In this, metabolism may be considered analogous to other complex processes: The steps that are rate-determining must be controlled in order to control output. Thus, regulation of metabolism under physiological conditions requires controlled flux through rate-limiting reactions, despite any changes in temperature or available substrate that might occur.

Current investigations suggest that rate compensation such as postulated above may indeed occur. An individual that is heterozygous at an enzyme locus has available two different forms of the enzyme; in the case of multimeric enzymes additional heterologous combinations occur. However, because substrate concentrations are usually very low under physiological conditions, the allozyme form having the lowest Michaelis constant, K_m (greatest affinity for substrate), will determine the reaction rate. Because K_m is a sensitive function of temperature and other variables, a small change in reaction conditions may significantly raise the K_m of the rate-determining form, lowering its activity. If this were the only enzyme available to catalyze the reaction, a marked change in reaction rate could result. The same change in reaction condition, however, may simultaneously lower the K_m of an alternative form, increasing its activity and thus minimizing the change in reaction rate. Such differences between alternative molecular forms, particularly with respect to sensitivity of substrate affinity to temperature, have been reported for both isozymes (*52*) and allozymes (*57*). Thus, individuals with multiple molecular forms of an enzyme may be capable of minimizing the effect of changed reaction conditions. Maximal activity would require a further compensation: Rearrangement of subunit composition, so that subunit α is not tied up in α/β heterodimer under conditions when the α/α homodimer is the physiologically important form. Such subunit exchange has been reported for isoenzymes (*58*) and may be demonstrated for allozymes in *Drosophila* (*57*).

Thus, individuals heterozygous at a locus may be capable of modulating reaction rate to compensate for variable reaction conditions. That such heterozygosity is associated predominantly with rate-limiting reactions in metabolism suggests an obvious selective role for these enzyme polymorphisms.

Among loci whose enzymes utilize substrates originating from the environment (substrates such as dietary proteins, alcohols, esters, or plant secondary compounds) considerable variation in available substrate type or concentration, or both, would be expected. Coordination of cellular metabolic pools requires that internal pool concentrations be buffered against such highly variable input. Such buffering might be achieved through multiple allelic forms of "external" enzymes having different substrate binding affinities. It might also be achieved by regulation of the amount of enzyme synthesized at dietary enzyme loci (*59*); regulatory modulation of transcription

Table 5. Enzyme polymorphism in man (*14*). Values of overall heterozygosity include the following additional loci for which monomorphism is reported: *Regulatory*—2,3-diphosphoglycerate mutase, hexokinase (red cell), malic enzyme (cytoplasmic), phosphofructokinase, pyruvate kinase; *Nonregulatory*—*N*-acetyl hexosaminidase, adenine phosphoribosyl transferase, catalase, glyoxalase II, guanase, guanine phosphoribosyl transferase, inosine triphosphatase, nucleoside phosphorylase, phosphoglycerate kinase, and pyrophosphatase.

Locus	HET
Variable substrate	
Acid phosphatases	0.17
Alkaline phosphatases	0.13
Esterases	0.05
Pepsinogen	0.47
Peptidases	0.08
Overall	0.18
Regulatory enzymes	
Acetylcholinesterase	0.23
Adenosine deaminase	0.11
Adenylate kinase	0.09
Alcohol dehydrogenase 1	0
Alcohol dehydrogenase 2	0.07
Alcohol dehydrogenase 3	0.48
Galactose-1-P uridyl transferase	0.11
Glyceraldehyde-3-P dehydrogenase	0
α-Glycerophosphate dehydrogenase	0
Glutamate-pyruvate aminotransferase	0.50
Hexokinase (white cell)	0.05
Malic enzyme*	0.30
Phosphoglucoisomerase	0
Phosphoglucomutase 1	0.36
Phosphoglucomutase 2	0
Phosphoglucomutase 3	0.38
Overall	0.13
Nonregulatory enzymes	
Amylase	0.09
Aldolase	0
Enolase	0
Glutamate-oxaloacetate aminotransferase†	0
Glutamate-oxaloacetate aminotransferase*	0.03
Isocitrate dehydrogenase†	0
Lactate dehydrogenase	0
Malate dehydrogenase†	0
Malate dehydrogenase*	0
6-Phosphogluconate dehydrogenase	0
Phosphoglyceromutase	0
Sorbitol dehydrogenase	0
Triose phosphate isomerase	0
Overall	0.005

* Mitochondrial. † Cytoplasmic.

and turnover rates has not been investigated extensively.

The role of allele multiplicity in polymorphism may be quite different from that of heterozygosity as such. The simplest hypothesis and, biochemically, the one which makes the most intuitive sense, is that polymorphic loci exhibiting many alleles reflect qualitative, as opposed to quantitative, variability in available substrates. Among genetic loci of enzymes utilizing externally originating substrates, far greater allele multiplicity is seen for insects than for either man or other vertebrates. Perhaps vertebrates are more restricted in the variety of their food supply than *Drosophila*, and thus experience less qualitative variability in the nature of proteins, alcohols, esters, and plant secondary compounds. Even within vertebrates, enzymes with broad specificities for internal metabolites may experience substantially more variation than enzymes with specific substrates, and higher numbers of alleles are indeed observed at the loci of such nonspecific enzymes. Polymorphic loci of enzymes utilizing specific internal metabolites rarely exhibit more than two alleles (*60*).

The suggested role of enzyme polymorphism in regulation bears directly upon several currently important issues in evolutionary biology. Selection for close linkage (supergenes) might involve evolutionary forces such as metabolic modulation by polymorphism, while "selectively neutral" polymorphisms certainly would not. The possible role of environmental variability in maintaining enzyme polymorphism may be understood in terms of metabolic modulation. More generally, the results I have discussed imply that the answer to the original question concerning the levels of gene variation depends upon the loci examined.

Where more than one locus participates in the regulation of a specific metabolic function, one might expect the necessity of regulatory coordination to influence degrees of polymorphism. The simplest solution to such a regulatory problem would be to avoid it by maintaining polymorphism at only one locus. Alternatively, close linkage of the joint regulatory loci would permit functional coordination of the allelic forms. This should result in detectable linkage disequilibrium. In most studies reported to date, joint regulatory interactions would not be expected; one might, however, expect regulatory interaction between enzyme pairs such as phosphoglucomutase and cytoplasmic ME (both are involved in determining the concentrations of NADPH available for oxidative metabolism). Only limited data on linkage are now available for enzyme loci in *Drosophila* (*61*); the possibility of linkage disequilibrium may be evaluated when more data become available.

Table 6. Metabolic patterns of polymorphism. Data are expressed as average heterozygosities.

Class of reaction	*Drosophila*	Small vertebrates	Man
Variable substrates	0.24	0.22	0.18
Specific substrates			
Regulatory	0.19	0.14	0.13
Nonregulatory	0.06	0.06	0.005
All loci	0.16	0.12	0.07

The relationships shown in Tables 3 to 5 argue strongly against the hypothesis that enzyme polymorphisms are selectively neutral. No hypothesis that maintains that polymorphic alleles are selectively equivalent can account for the relationship that clearly exists between enzyme polymorphism and enzyme function. Other general arguments against selective neutrality have relied primarily on data from those few loci with large numbers of alleles (*23*). The relationship of heterozygosity to metabolic function, by contrast, is equally apparent for loci with only two or three alleles; this argues that polymorphism is influenced by selection generally, rather than the multi-allele loci constituting a special case.

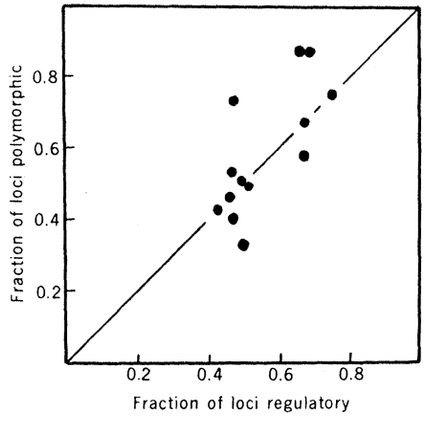

Fig. 1. Relation of incidence of polymorphism among *Drosophila* to enzyme regulatory character. Data are from those cited in Table 3. Each point represents a different species of *Drosophila*; the solid line represents the axis of identity. Incidence of polymorphism is scored as the occurrence of two or more alleles at frequencies ≥ 0.01 in at least half of all populations sampled for a minimum of 50 genes. Loci of enzymes utilizing nonspecific substrates are not considered. The correlation is significant ($R = 0.72, F = 12.2, P < .01$).

It has often been suggested that environmental heterogeneity should have an important influence upon degrees of enzyme polymorphism (*54*). This would be expected if enzyme polymorphism indeed reflected a strategy of metabolic compensation. According to this hypothesis, the basis of the selective advantage of heterozygotes lies in the sensitivity of regulatory enzymes to reaction conditions. Major regulatory reactions are rate-limiting precisely because the enzymes catalyzing them have low capacities; outside of a rather narrow range of conditions, their activity may be affected by alterations in temperature or other variables. Enzymes fully active over a broad range of conditions are not expected to be rate-limiting because of their high enzyme capacities. Thus in a heterogeneous environment the optimal evolutionary strategy for regulatory reactions is to have alternative enzyme forms available, rather than a single form of high capacity. This suggests that the critical factor in determining the relative fitness of individuals heterozygous at an enzyme locus is the probability of their having to contend with widely divergent reaction conditions. In a constant environment, the optimal form of the enzyme will be the best-functioning homodimer, while in an environment where the probability of divergent reaction conditions is greater, heterozygous individuals may be at an advantage. Thus, clines in environmental variability may be responsible for the biogeographic clines in heterozygosity often reported at enzyme loci. An example of an enzyme with a typical cline is alcohol dehydrogenase (ADH) in *D. melanogaster*. In Florida ADH is essentially monomorphic; heterozygosity at this locus increases with latitude, with maximal possible heterozygosity being maintained at the northern limits of its distribution (*19*).

It is important to distinguish between variability and predictability. According to the hypothesis I have outlined, degrees of heterozygosity will reflect the probability of variable reaction conditions. Thus, the effect of introducing a stochastic element into the pattern of variation, rendering it less predictable, will depend upon this probability. When a relatively constant environment is rendered less predicta-

ble, the effect is to increase variability and favor heterozygotes. When a predictably variable environment is rendered less predictable, the effect may be to increase the probability that an individual will encounter constant conditions, thus reducing the advantage of the heterozygote. The selectively important parameter will be the degree of variability actually encountered.

In recent years, many attempts have been made to estimate amounts of overall genetic variability. The amounts reported to date have been remarkably constant from one species to another (*14*, *62*). The variability is usually characterized by the average heterozygosity observed among a variety of randomly chosen enzyme loci. The relation of polymorphism to enzyme function suggests that a better approach would be to compare variability within enzyme classes. Such a comparison is presented in Table 6. The *Drosophila* and small vertebrate species considered exhibit considerable overall variability within groups; the values for overall mean heterozygosity shown in Table 6 are thus somewhat oversimplified. For instance, among the 13 *Drosophila* species reported in Table 3, a bimodal distribution of overall average heterozygosity is suggested; within each of the two modes, however, variability is remarkably consistent. Evaluation of polymorphisms by enzyme class reveals the underlying pattern of variation. Among loci of enzymes experiencing highly variable substrate concentrations, similar amounts of variability occur in all three groups of organisms. Among regulating loci, reported variability is again about the same in insects, man, or small vertebrates. Among nonregulatory loci, however, the amount of variability in human beings is far less than in either small vertebrates or insects! It is this difference that has indicated that the human genome has a lower level of variability (*14*, *63*). Table 6 suggests that loci not under strong selection for polymorphism are less variable in man, perhaps reflecting his population structure or evolutionary history. In summary, amounts of variation at enzyme loci do seem similar in different organisms; however, certain classes of enzyme loci are exceptions.

One must approach with caution estimates of genetic variability which are phrased in terms of specific selections of loci, such as estimates based upon the proportion of the genome observed to be polymorphic. It seems clear that the estimate obtained depends upon which loci are chosen for examination. In the studies of *Drosophila* cited in Table 3, the fraction of "specific substrate" loci observed to be polymorphic seems closely related to the relative proportions of regulatory and nonregulatory loci chosen for examination (Fig. 1).

Although the observed patterns of enzyme polymorphism suggest that they may have regulatory significance, this hypothesis must be verified by detailed biochemical experimentation. Although researchers are actively engaged in such experimentation, few data are yet available.

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