

LIMITS OF ADAPTATION: THE EVOLUTION OF SELECTIVE NEUTRALITY

DANIEL L. HARTL, DANIEL E. DYKHUIZEN AND ANTONY M. DEAN

Department of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110

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ABSTRACT

Many enzymes in intermediary metabolism manifest saturation kinetics in which flux is a concave function of enzyme activity and often of the Michaelis-Menten form. The result is that, when natural selection favors increased enzyme activity so as to maximize flux, a point of diminishing returns will be attained in which any increase in flux results in a disproportionately small increase in fitness. Enzyme activity ultimately will reach a level at which the favorable effect of an increase in activity is of the order $1/(4N_e)$ or smaller, where N_e is the effective population number. At this point, many mutations that result in small changes in activity will result in negligible changes in fitness and will be selectively nearly neutral. We propose that this process is a mechanism whereby conditions for the occurrence of nearly neutral mutations and gene substitutions can be brought about by the long-continued action of natural selection. Evidence for the hypothesis derives from metabolic theory, direct studies of flux, studies of null and other types of alleles in *Drosophila melanogaster* and chemostat studies in *Escherichia coli*. Limitations and complications of the theory include changes in environment or genetic background, enzymes with sharply defined optima of activity, overdominance, pleiotropy, multifunctional enzymes and branched metabolic pathways. We conclude that the theory is a useful synthesis that unites many seemingly unrelated observations. The principal theoretical conclusion is that the conditions for the occurrence of neutral evolution can be brought about as an indirect result of the action of natural selection.

THE neutral theory of molecular evolution (KIMURA 1968) has provided much insight for understanding the rates and patterns of change in macromolecules through evolutionary time, and also for understanding the role of mutation and random genetic drift in the maintenance of contemporary genetic polymorphisms (KIMURA 1983a,b). At the same time the theory has been highly controversial. In some commentaries the neutral theory is even regarded as anti-Darwinian. However, as has been demonstrated in an epigraph (DYKHUIZEN and HARTL 1980), Darwin was well aware of the possibility of selectively neutral variants and evidently did not consider their existence a challenge to his theory.

When considered from a certain perspective, the superficial inconsistency between Darwinian selection and neutral evolution disappears completely. In-

deed, the theories are not only consistent, but there is an important connection between the theories that we propose to explore in this paper. We shall develop arguments based on considerations of general characteristics of metabolic enzymes. Among the class of gene products for which the considerations are valid, the rather surprising conclusion is that the long-continued action of Darwinian natural selection may, itself, result in a situation in which many mutations are selectively neutral or nearly neutral.

SATURATION KINETICS IN METABOLIC PATHWAYS

Many biochemical reactions and metabolic pathways exhibit some type of saturation kinetics in which the reaction velocity or flux through the pathway converges asymptotically to a plateau as the activity of an enzyme increases, until a point is reached after which a further increase in enzyme activity results in a negligible increase in reaction velocity or flux (KACSER and BURNS 1973; CROWLEY 1975; ALBERY and KNOWLES 1976; CORNISH-BOWDEN 1976; PRICE and STEVENS 1982). Saturation behavior is expected in cases in which reaction velocity or flux is limited by substrate availability (MICHAELIS and MENTEN 1913; BRIGGS and HALDANE 1925), or in linear metabolic pathways with fixed inputs of the type studied by KACSER and BURNS (1973, 1979). Saturation kinetics also provides the framework for the molecular theory of dominance (WRIGHT 1934; KACSER and BURNS 1981), and it justifies the assumption that fitness functions are generally concave (GILLESPIE 1976; MIDDLETON and KACSER 1983; WATT 1985).

With regard to the modulation and control of complex metabolic networks, many of the corollaries of saturation kinetics have been deduced (KACSER and BURNS 1973, 1979; HEINRICH and RAPOPORT 1974, 1983), and the development of a general theory of metabolic processes is regarded as being of profound importance in cell biology and physiology (PORTEOUS 1983). However, saturation kinetics also has important implications for the evolution of enzymes and of entire metabolic pathways, and several of the implications run contrary to intuitive notions but are supported by observed data. The implications of saturation kinetics for molecular evolution are considered in this paper. Although the arguments will be discussed primarily in the context of enzyme function and evolution, the ideas are supposed to apply rather more generally to structural and regulatory gene products.

THE SATURATION MODEL

Saturation kinetics may be illustrated by a function of the type in Figure 1. The exact shape of the curve is largely immaterial to the arguments to be developed, and we shall use an equation of the type $F = AF_{\max}/(K_A + A)$, where F represents the flux through a metabolic pathway (or rate of reaction), and A represents the amount (or activity) of an enzyme in the pathway. The quantities F_{\max} and K_A represent, respectively, the maximum possible value of flux and the amount of enzyme at which flux attains one-half of its maximum value. The two-parameter saturation equation follows from simple MICHAELIS-MEN-

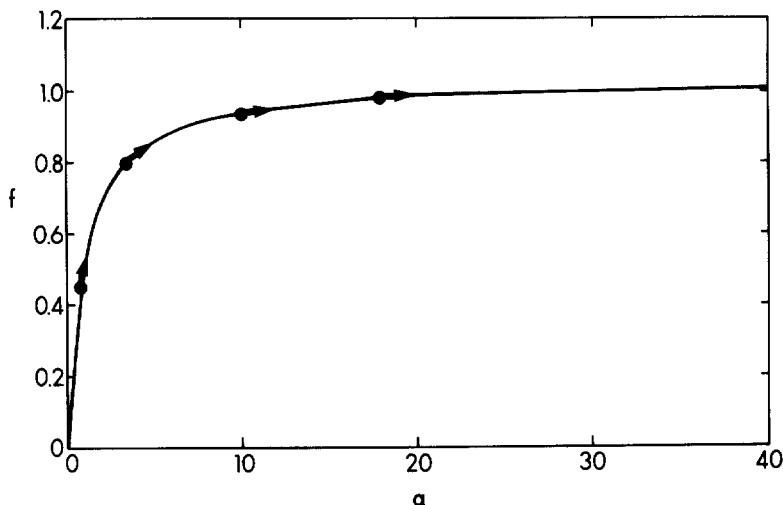


FIGURE 1.—Standardized Michaelis-Menten saturation equation $f(a) = a/(1 + a)$ scaled to equal 1 at $a = 30$.

TEN (1913) or BRIGGS-HALDANE (1925) enzyme kinetics when A is limiting to flux; it appears in the MONOD (1950) model of bacterial growth in a chemostat, and similar kinds of equations emerge from theoretical analysis of multienzyme metabolic pathways (KACSER and BURNS 1973, 1979; SAVAGEAU 1976).

Defining $f = F/F_{\max}$ and $a = A/K_A$ yields a parameter-free version of the saturation equation, namely $f = a/(1 + a)$, which is the curve illustrated in Figure 1. The curve rises sharply at first, but then increases more gradually to a plateau. We suppose a situation in which the fitness (f) of an organism is proportional to flux and can therefore be represented by means of a saturation curve, with the quantity a representing the amount of an enzyme or other gene product. Concave fitness functions of the general type in Figure 1 have been assumed by WRIGHT (1934), GILLESPIE (1976) and KACSER and BURNS (1981). The genotype of an organism (or an entire population) may be represented as a point on the curve, and barring such phenomena as overdominance, natural selection is expected to operate in such a manner that a population moves gradually upward on the curve, as illustrated by the arrows. The following sections concern the implications of natural selection when evolution occurs along the trajectory of a saturation curve.

ASYMMETRY OF SELECTIVE EFFECTS

Figure 2 illustrates the selection coefficient resulting from a mutation that changes a by a fixed positive or negative amount in the case $a_0 = 30$. For a change in a from a_0 to a_1 , the selection coefficient is given by $s = 1 - [f(a_0)/f(a_1)]$, with s greater than or less than 0 as a_1 is greater or less than a_0 , respectively. For a fixed but small $\Delta a = a_1 - a_0$, the selection coefficient is equal in magnitude but opposite in sign because the $f(a)$ is continuous and differentiable. However, for Δa not infinitesimal, the magnitude of the selec-

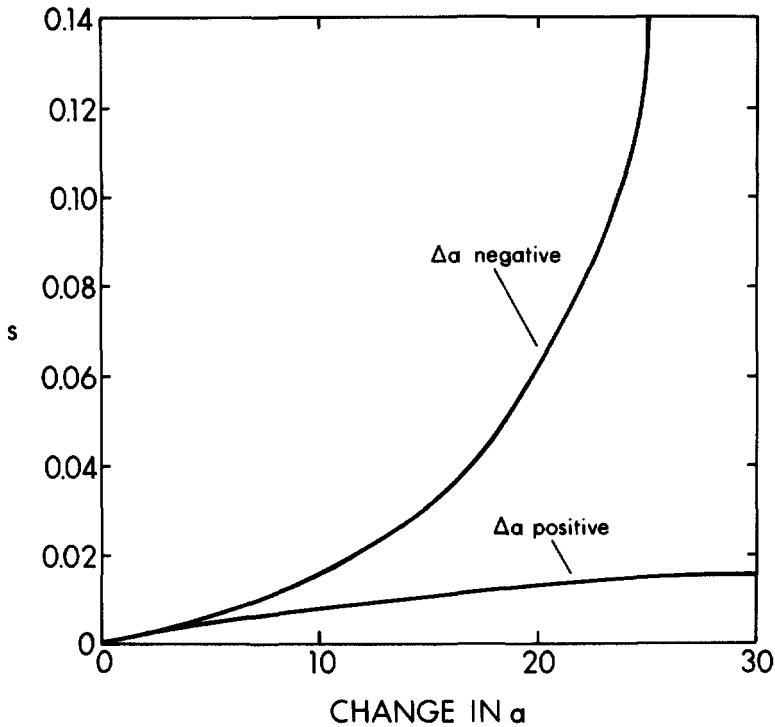


FIGURE 2.—Selection coefficient (s) resulting from a given positive or negative change in enzyme activity (Δa), when initially $a = 30$.

tion coefficients will generally be different. In particular, enzymes that have evolved to the plateau in Figure 1 will have the characteristic that a decrease in enzyme activity will result in a greater decrease in fitness than an identical increase in enzyme activity.

The principle illustrated in Figure 2 accounts, in part, for the reason that harmful mutations are generally detected more frequently than favorable mutations. On an *a priori* basis it seems reasonable to assume that random changes in amino-acid sequence will more often impair enzyme performance than improve it. However, Figure 2 indicates that, in addition to the *a priori* argument, detrimental mutations will be observed more often than favorable ones because a decrease in activity will result in a greater effect on the phenotype.

DIMINUTION OF SELECTIVE EFFECTS

For the relation in Figure 1, the increment in fitness for a given small increase in enzyme activity is $df/da = 1/(1+a)^2$, or approximately $1/a^2$ to a good approximation for populations that have evolved out onto the plateau. If $a = 100$, for example, the increment in fitness resulting from a one-unit increase in enzyme activity will be approximately $s = 10^{-4}$. (The exact value is $s = 1/a(a+2) = 9.8 \times 10^{-5}$.) Thus, the favorable effects of an increase in enzyme activity diminish rapidly as an enzyme improves under the joint effects

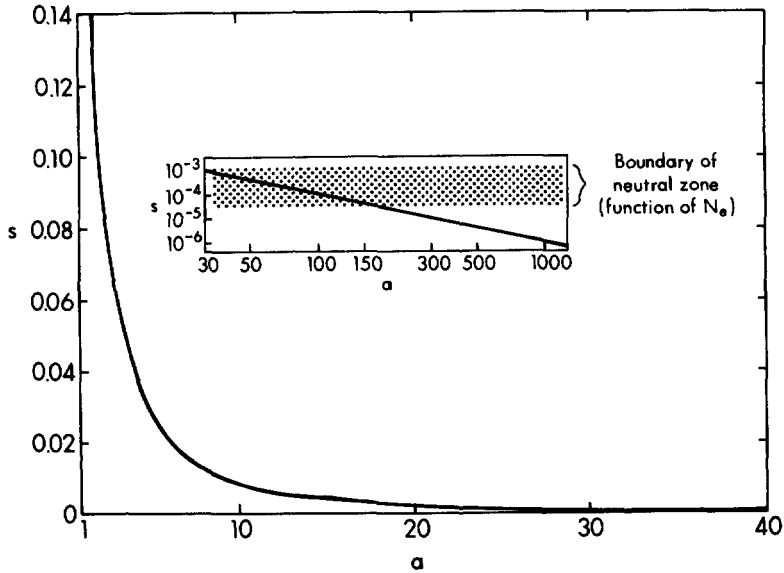


FIGURE 3.—Selection coefficient (s) resulting from an increase in a single unit of enzyme activity when the initial activity equals a . Inset shows the tail of the curve on a logarithmic scale and the critical region in which $s \approx 1/(4N_e)$. The function is $s = 1/[a(a + 2)]$.

of mutation and natural selection. This implication of Michaelis-Menten kinetics has also been pointed out by CORNISH-BOWDEN (1976).

Figure 3 illustrates the diminution of the selective advantage resulting from a unit increase in enzyme activity, using as a specific example the curve in Figure 1. As an enzyme improves as a result of the action of natural selection, each additional increment in enzyme activity results in a smaller net gain in fitness.

THE NATURAL SELECTION OF SELECTIVE NEUTRALITY

In an infinite population, favorable mutations would be expected to become fixed in sequence, driving the population ever further along the plateau in Figure 1. However, a most interesting implication of Figure 3 occurs in the context of a finite population sufficiently small in size that random genetic drift becomes important. As Kimura has shown (KIMURA 1964; KIMURA and OHTA 1971), mutations behave as if they were selectively neutral in a diploid population when the quantity $4N_e s$ is sufficiently smaller than 1. Roughly speaking, natural selection will dominate the fixation process when $4N_e s$ is greater than 10, random drift will dominate the fixation process when $4N_e s$ is smaller than $1/10$, and both processes will play a role for intermediate values of $4N_e s$. In a haploid population the corresponding quantity is $2N_e s$. The implication of Figure 1 is that, eventually, under the long continued pressure of natural selection for enzyme improvement, an enzyme will reach a state of diminishing favorable effects in which many new mutations affecting enzyme activity result in negligible changes in fitness (that is, in which $4N_e s \ll 1$).

In terms of Figure 3, the evolution of selective neutrality implies that there is a value on the ordinate corresponding to $1/4N_e$ around which the selective values begin to blend into effective neutrality and toward which natural selection tends to evolve the enzyme. The region of the curve bounded by $1/4N_e$ may be called the neutral zone of enzyme evolution because mutations with selective values within this zone are affected by random genetic drift, as well as by selection. The fate of mutations with s much smaller than $1/4N_e$ is determined primarily, if not exclusively, by random genetic drift. In Figure 3, the inset shows the tail of the curve, plotted on a logarithmic scale, with the boundary of the neutral zone shown as indistinct because the demarcation is a function of N_e . However, the boundary is not sharp even when N_e is known, because as s approaches and becomes smaller than $1/4N_e$, the role of selection in determining the fate of a mutation very gradually diminishes, whereas the role of random genetic drift gradually becomes more prominent. The transition in selection coefficients between "selected" alleles and "neutral" alleles is gradual.

The asymmetry in Figure 2 also plays a role in evolution within the neutral zone because mutations of equal but opposite effect result in a greater diminution than gain in fitness. Because *a priori* most mutations would be expected to be unfavorable for enzyme activity, most mutations that are fixed by random genetic drift would be expected to be very slightly deleterious, as OHTA (1976) has suggested.

Because small changes in kinetic parameters or enzyme amount are generally difficult to detect without special procedures, the effects of random amino-acid substitutions on enzyme activity are largely unknown. However, the arguments presented regarding the evolution of selective neutrality imply that many amino-acid substitutions may be located within the neutral zone in spite of the fact that they may change enzyme activity to some, perhaps biochemically significant, extent.

The saturation theory of molecular evolution is not incompatible with the existence of selective constraints. Our theory deals with the relation between enzyme activity and fitness, whereas selective constraints deal with the relation between mutations and enzyme activity. When an enzyme, or part of an enzyme (such as the region around the active site) is so constrained in amino-acid sequence that virtually all mutations result in large decreases in activity, then neutral or nearly neutral mutations cannot occur.

EVIDENCE FOR THE SATURATION THEORY

Evidence in support of the saturation theory may be derived from several sources, each of which warrants a brief discussion.

Evidence from metabolic theory: KACSER and BURNS (1973) define the sensitivity coefficient of an enzyme as the ratio of proportional changes in f and a , which is $Z = (df/f)/(da/a) = 1/(1 + a)$. The sensitivity coefficient gives the proportional change in flux through a pathway as a function of the proportional change in enzyme activity. A sensitivity coefficient of unity implies that change in flux is proportional to change in enzyme activity. KACSER and BURNS (1973,

1979) make the key observation that, in segments of complex metabolic pathways, the sensitivity coefficients must sum to unity. An analogous summation principle governs the transients in coupled enzyme systems (EASTERBY 1973). The summation principle implies that sensitivity coefficients must, in general, be smaller than unity. A sensitivity coefficient of less than unity implies that a small change in the activity or amount of an enzyme will result in an even smaller change in flux, which, in turn, will result in a still smaller change in fitness. On the other hand, in those rare cases in which the activity of a particular enzyme in a pathway is the limiting factor in flux, then the sensitivity coefficient of all other enzymes in the pathway must be very small. The principle of shared controls in simple metabolic pathways appears to be quite general (KACSER and BURNS 1973, 1979; SAVAGEAU 1976), but the behavior of complex branched and interconnected pathways is still undergoing analysis.

Evidence from direct studies of flux: KACSER and BURNS (1973, 1981) have provided numerous examples in which the observed relation between metabolic flux and enzyme amount (or activity) exhibits the general shape depicted in Figure 1. Additional examples include metabolic flux in the arginine biosynthetic pathway in *Neurospora crassa* (FLINT, PORTEOUS and KACSER 1980; FLINT *et al.* 1981) and ethanol flux in relation to alcohol dehydrogenase activity in *Drosophila melanogaster* (MIDDLETON and KACSER 1983). In most examples, a reduction of 50% in enzyme amount results in little or no reduction in flux.

Evidence from alcohol dehydrogenase in Drosophila: As noted, in many cases the observed relation between metabolic flux and enzyme activity follows the general pattern depicted in Figure 1. However, the relation between *fitness* and enzyme activity is more difficult to examine directly, although most evolutionary considerations of metabolic flux are predicated on the sometimes implicit assumption that fitness is directly proportional to flux (GILLESPIE 1976; KACSER and BEEBY 1984). One carefully studied case is that of alcohol dehydrogenase in *D. melanogaster*, in which all genotypes (excluding homozygotes for a null allele) exhibit the same survivorship when exposed to ethanol (MIDDLETON and KACSER 1983, table 5).

A second line of evidence relative to the relation between alcohol dehydrogenase activity and fitness may be derived from the frequency of null alleles in natural populations. The null allele frequency of *Adh* (British and American studies combined) is $1/1170 = 8.55 \times 10^{-4}$ (LANGLEY *et al.* 1981). We assume a mutation rate to *Adh* nulls of 3.2×10^{-6} [data of MUKAI and COCKERHAM (1977) as corrected by VOELKER, SCHAFFER and MUKAI (1980)]. Assuming mutation-selection equilibrium, the estimated fitness of the *Adh* null heterozygote is therefore $1 - hs = 1 - \mu/q = 1 - (3.2 \times 10^{-6}/8.55 \times 10^{-4}) = 0.9963$, where μ is the mutation rate to null alleles, h is their degree of dominance, and q is their equilibrium frequency. Since heterozygotes for *Adh* null alleles demonstrate half the enzyme activity of wildtype homozygotes (MIDDLETON and KACSER 1983) a reduction in enzyme activity by a factor of two reduces the fitness by 0.0037. Putting $f(a/2) = 0.9963$ in the standardized saturation equation results in $a = 538.5$. Thus, an increase in *Adh* activity of 1% would result in a selection coefficient, relative to wildtype, of $f(543.9)/f(538.5) - 1$

TABLE 1

Null alleles in Drosophila

Enzyme locus	$q \times 10^3$	$hs \times 10^3$	a	t
<i>Got-2</i>	2.63	1.50	1331.3	7.4×10^{-6}
α <i>Gpdh</i>	8.33	0.46	4345.8	2.3×10^{-6}
<i>cMdh</i>	1.64	2.36	845.4	1.2×10^{-5}
<i>Adh</i>	0.85	4.52	440.5	2.2×10^{-5}
<i>Dip-A</i>	4.36	0.89	2245.2	4.4×10^{-6}
<i>Pgi</i>	0.87	4.45	442.4	2.2×10^{-5}
<i>Hex-C</i>	1.72	2.25	886.9	1.1×10^{-5}
<i>Idh</i>	2.20	1.76	1134.4	8.7×10^{-6}
<i>Pgm</i>	0.74	5.19	383.4	2.6×10^{-5}
<i>Est-C</i>	5.15	0.75	2664.7	3.7×10^{-6}
<i>Odh</i>	0.85	4.53	439.5	2.2×10^{-5}
<i>Men</i>	6.10	0.63	3172.6	3.1×10^{-6}
<i>Aldox</i>	11.30	0.34	5880.4	1.7×10^{-6}
<i>Acp-1</i>	2.47	1.57	1271.9	7.8×10^{-6}

Allele frequencies q of null alleles in *Drosophila* (assumed equilibrium), combined American and British data from LANGLEY *et al.* (1981). Fitness reduction in heterozygotes hs calculated from average null mutation rate of VOELKER, SHAFFER and MUKAI (1980). Values of a are those in wild-type homozygotes, assuming the fitness function in Figure 1, and values of t correspond to the theoretical selection coefficients resulting from an increase in enzyme activity of 1%.

$= 1.8 \times 10^{-5}$. Indeed, an increase in *Adh* activity of 10% would result in a selection coefficient of only 1.7×10^{-4} . (In general, an increase in enzyme activity by 100x percent will result in a favorable selection coefficient of $t = x/(a + ax + 1)$, or roughly x/a when a is large and x is small.) If these considerations are valid, then it must be inferred that wild-type *Adh* activity is well within the neutral zone.

Evidence from frequencies of null alleles in D. melanogaster: The calculation of a performed with *Adh* in the previous section is extended to other enzymes in Table 1, the null frequencies in which are taken from LANGLEY *et al.* (1981). The mutation rate assumed is 3.86×10^{-6} (VOELKER, SHAFFER and MUKAI 1980), and it can be shown that, when $f(a/2) = 1 - hs$, then $a = 2[(1/hs) - 1]$. The column headed t gives the selection coefficients resulting from a 1% increase in enzyme activity in the wild-type genotypes. The predicted selection coefficients range from 1.7×10^{-6} to 2.6×10^{-5} , with a median of 9.5×10^{-6} . For all the genes in Table 1, the average null allele frequency is 2.45×10^{-3} , giving an average $hs = 0.0015$ (LANGLEY *et al.* 1981) and an average $a = 1331$. With this value of a , a 1% increase in activity results in $t = 7.4 \times 10^{-6}$, and a 10% increase in activity results in $t = 6.8 \times 10^{-5}$. Assuming an effective size of a *Drosophila* deme in North America on the order of 10^4 (MUKAI and YAMAGUCHI 1974; MUKAI and NAGANO 1983), many mutations that change activity by a small amount will have their fate determined largely, if not exclusively, by random genetic drift. Selection for enzyme activity would

be still weaker in Japan, where the effective size of *Drosophila* demes is on the order of 10^3 (KUSAKABE and MUKAI 1984).

Evidence from detrimental and lethal genes in D. melanogaster: The heterozygous viability of lethals maintained in natural populations of *Drosophila* ranges between 0.97 and 0.99 (SIMMONS and CROW 1977). Uncharacterized lethal alleles are rather more difficult to interpret biochemically than null alleles. Undoubtedly, some lethals result from simple null mutations in genes with essential functions; however, it is probable that many lethals also result from more complex genetic changes, including the insertion of transposable elements. Whatever their molecular basis, if one deliberately simplifies the situation by assuming that lethals represent a class of mutations that eliminate gene activity, then the fitness of lethal heterozygotes corresponds to $f(a/2)$, as with the simple null alleles of enzyme loci. For $hs = 0.01-0.03$, the range of $a = 65-200$, which, for a change in a of 1%, results in a selection coefficient of 1.5×10^{-4} to 4.9×10^{-5} . If this kind of calculation is valid, then it would imply that small changes in the activity of essential genes results in disproportionately small changes in fitness.

MUKAI *et al.* (1972) and OHNISHI (1977) have shown that the degree of dominance of detrimental mutations in *Drosophila* is approximately $h = 0.30-0.50$, which is much greater than the degree of dominance of lethals (reviewed in SIMMONS and CROW 1977). GILLESPIE (1978) has drawn attention to the fact that the differing degrees of dominance imply that the fitness function must be concave, and he illustrates an example similar in shape to the curve in Figure 1 (GILLESPIE 1976, page 817). Assuming an additive activity scale (GILLESPIE 1978) and a simple Michaelis-Menten relation between fitness and activity, the average values of $s = 0.09$ and $h = 0.40$ for detrimental mutations summarized by SIMMONS and CROW (1977) may be shown to imply that the a for wild-type homozygotes is 43.5, and that the increased fitness resulting from a 1% increase in activity in such a locus is approximately 2.2×10^{-4} .

Evidence from 6PGD in Escherichia coli: DYKHUIZEN and HARTL (1980) have described two naturally occurring allozyme alleles of the *gnd* locus coding for 6-phosphogluconate dehydrogenase, which differ by 27% in their K_m values for substrate and yet are selectively equivalent ($s < 0.002$ per hr) in chemostats in which the limiting nutrient is either glucose or gluconate. However, the same alleles exhibit selection in gluconate-limited chemostats when the genetic background contains an *edd*⁻ mutation, which blocks an alternative metabolic pathway for gluconate metabolism. This example shows not only that an observed difference in enzyme activity need not result in a difference in fitness but also that the relation between fitness and enzyme activity depends upon the environment and the genetic background.

A second example of a large activity difference in 6PGD resulting in a negligible difference in fitness concerns the *gnd*⁺(862) allele detected as a mutation in a strain of genotype *gnd*⁺ *edd*⁻ that took over in a chemostat limited for gluconate (MILLER *et al.* 1984). The evolved allele, which results from a specific deletion upstream from the coding region, exhibits a 300% increase in the amount of 6PGD but only a 57% increase in the maximum

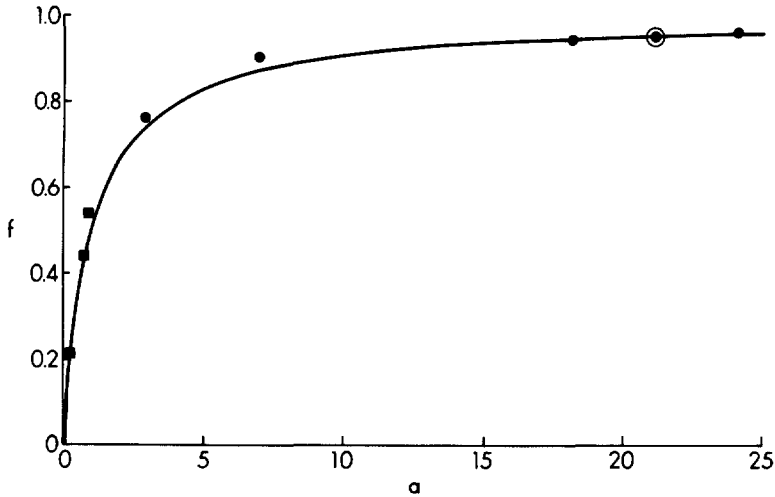


FIGURE 4.—Growth rate in lactose medium vs. lactase activity in *E. coli*, scaled as in Figure 1. Dots and squares represent the *lacZ* and *ebg* β -galactosidase enzymes, respectively. The circled dot represents *E. coli* K12. Lactase activities per milligram of purified *ebg* protein were calculated relative to *lacZ* β -galactosidase according to data in HALL (1982, 1984) and HUBER, KURZ and WALLENFELS (1976). Relative activities of *lacZ* β -galactosidases were based on the ratio of K_m values toward *o*-nitrophenyl- β -D-galactopyranoside (ONPG). All points pertain to growth rates when the *lac* operon is fully induced or in constitutive mutants.

growth rate in gluconate medium. Moreover, in chemostats in which the limiting nutrient is glucose, the high-activity *gnd* allele has no detectable selective advantage (D. E. DYKHUIZEN, unpublished observations).

Evidence from β -galactosidase in E. coli: We have studied the relation between fitness and activity of β -galactosidase in mutants of *E. coli* K12. The fitness of one strain, relative to another, is measured in terms of the magnitude of selection that occurs when the strains are in direct competition in a chemostat in which the limiting nutrient is lactose. Data derived from nine mutants are shown in Figure 4, where both axes are scaled as in Figure 1. The points denoted by squares were calculated from published studies of *ebg* [evolved β -galactosidase, studied by HALL (1982, 1983, 1984)], and the remaining points were determined by us from chemostat studies of β -galactosidase mutants characterized and provided by LANGRIDGE (1974) or from newly obtained β -galactosidase mutants. The observed fitness-activity data fit a saturation curve very well.

In terms of the unstandardized Michaelis-Menten equation, the parameters are $F_{\max} = 1.036$ and $K_A = 0.041$. These values imply that a hypothetical mutation that increased β -galactosidase activity by 1% would result in a fitness increase of 3.6×10^{-4} relative to *E. coli* K12. However, the fitness relation in Figure 4 pertains to the most intense form of competition for lactose to which cells of *E. coli* can be subjected; namely, competition for lactose when lactose is the sole nutrient limiting to growth. The relation in Figure 4 is therefore an upper bound to the type of relation that would exist under natural conditions in which competition for lactose is rarely, if ever, so intense.

POTENTIAL COMPLICATIONS AND LIMITATIONS OF THE THEORY

Although the saturation theory of molecular evolution would appear to apply to many enzymes, and there is much evidence to support the theory in several individual examples, many individual exceptions also exist. The types of situations in which exceptions might occur are described briefly as follows:

Overdominance in activity: When there is interaction between the enzyme subunits produced by alternative alleles in a heterozygous individual, then it is theoretically possible that the overall enzyme activity in the heterozygous individual could be greater than that in either of the homozygotes, resulting in overdominance in fitness. If the activities of the homozygous genotypes are already far out on the plateau in Figure 1, then overdominance is of little relevance, because all of the differences in fitness may be located within the neutral zone. However, when overdominance occurs in the region of Figure 1 near the shoulder, then neither allele will be able to become fixed, and it will be difficult for the population to move up the curve toward the plateau.

GILLESPIE (1978) has considered a model of randomly fluctuating environments predicated on a fitness-activity relation similar to that in Figure 1, and in which the enzyme activity of homozygous genotypes fluctuates randomly in time and space. His analysis shows that, when alleles are additive on the activity scale and the fitness function is concave, then polymorphisms can be maintained by fluctuating selection (SAS-CFF model). However, the polymorphism results in essence from a sort of marginal overdominance in the heterozygotes, and hence the model cannot be invoked in the case of haploid organisms such as *E. coli*, which are highly polymorphic for allozymes (MILKMAN 1973; SELANDER and LEVIN 1980; WHITTAM, OCHMAN and SELANDER 1983). The marginal overdominance resulting from stochastic selection may be significant when the enzyme activities are sufficiently near the shoulder in Figure 1. However, it may be inferred from the data in Table 1 for null alleles and from the comparable calculations for recessive lethal and detrimental alleles in *Drosophila* that the enzyme activities in wild-type genotypes are generally rather far out along the plateau.

Fluctuations in the environment: In some cases the functional relation between fitness and enzyme activity may be significantly affected by the environment or the genetic background of the cell or organism. As noted in connection with the evolved *gnd* allele *gnd*⁺(862), selection in favor of the mutant is evidenced in chemostats when the limiting nutrient is gluconate, but not when the limiting nutrient is glucose. This result is interpreted in terms of Figure 1 as implying that the fitness-6PGD activity relation differs according to whether growth is limited by gluconate or glucose. In these terms the activity of the *gnd*⁺(862) allele results in a greater fitness than the wild-type allele relative to the gluconate curve, but the activity of both *gnd*⁺(862) and the wild-type allele are located well onto the fitness plateau of the glucose curve. Displacement or change in shape of the fitness-activity relation is one possible mechanism resulting in the phenomenon we have previously called the potential for selection of otherwise selectively neutral alleles (DYKHUIZEN and HARTL 1980, 1983;

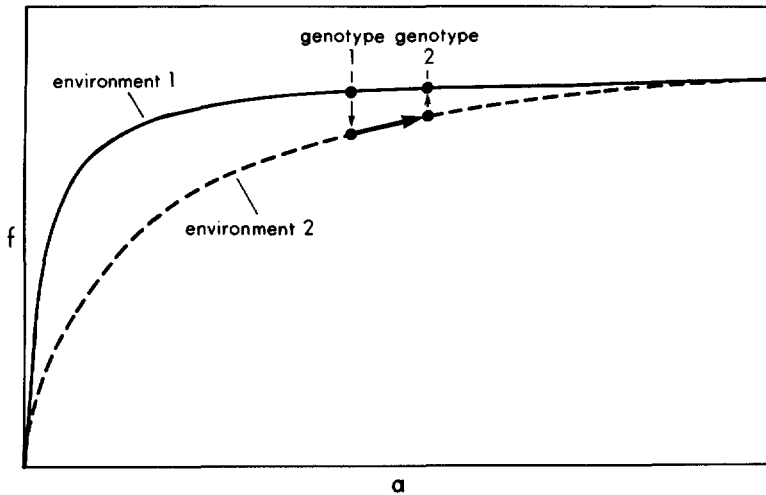


FIGURE 5.—Hypothetical example in which the fitness function changes, and selection occurring in the unfavorable environment 2 can increase enzyme activity beyond the point that would be likely to occur as a result of the random genetic drift of nearly neutral mutations in the favorable environment 1.

HARTL and DYKHUIZEN 1981, 1985; DYKHUIZEN, DE FRAMOND and HARTL 1984). In general, when a potential for selection among nearly neutral alleles exists, one expects the potential to be realized under unusual or stressful environmental conditions. Thus, for example, although it has been demonstrated that selection does occur among *Adh* alleles in *Drosophila* grown in medium containing high concentrations of ethanol or other alcohols (VAN DELDEN, KAMPING and VAN DIJK 1975; VAN DELDEN, BOERMA and KAMPING 1978), this observation does not necessarily imply that the alleles will also be selected under less stressful conditions, because the relation between fitness and enzyme activity undoubtedly depends, in part, on the type and concentration of alcohol in the environment.

When the environment fluctuates in such manner as to alter the fitness-activity relation, and when high enzyme activity is beneficial to fitness in one environment but harmful in another, then the overall result of natural selection may be selection for an optimum enzyme activity or, perhaps, a polymorphism.

Changes in the fitness-activity relation resulting from alternative environments or genetic backgrounds provide one mechanism by which enzyme activity can evolve to a location further along the fitness plateau than would be likely in a constant environment. The mechanism is illustrated in Figure 5. Fluctuations between environment 1 and environment 2 result in the fixation of genotype 2, even though the genotypes are selectively nearly neutral in environment 1. Genotype 2 is also ultimately fixed if the two environments occur in alternate patches in space. Natural selection ultimately results in genotypes that produce sufficiently high enzyme activities that fitness is effectively buffered against the effects of random genetic or environmental perturbations in enzyme activity. One result of this phenomenon is the evolution of domi-

nance at the level of phenotype (KACSER and BURNS 1981). The high values of a calculated for null enzyme alleles (Table 1), recessive lethal alleles, and detrimental alleles in *Drosophila* suggest a very significant buffering effect, which not only buffers against random environmental fluctuations but also against small perturbations in enzyme activity resulting from new mutations within the genes themselves.

Coordinate evolution of functionally linked enzymes: A potential complication in the theory occurs because the evolution of any enzyme within a metabolic pathway must necessarily affect the relation between fitness and the activity of other enzymes in the same pathway, thus changing the fitness function. Natural selection will inevitably have the greatest effect upon the enzymes in a pathway that possess the largest sensitivity coefficients, which implies that the remaining enzymes must possess correspondingly small sensitivity coefficients placing them farther into the neutral zone in Figure 3. However, the eventual result of natural selection upon an enzyme that possesses a high sensitivity coefficient must be a decrease in the sensitivity coefficient. Since the sensitivity coefficients of the enzymes in a pathway must sum to unity (KACSER and BURNS 1973, 1979), a decrease in the sensitivity coefficients of one or more enzymes implies an increase in the sensitivity coefficients of other enzymes in the same pathway. Thus, the focus of natural selection shifts, and an enzyme that was previously far out on the curve in Figure 1 (low sensitivity coefficient) may be displaced onto the shoulder (high sensitivity coefficient). In other words, alternate forms of an enzyme that are selectively nearly neutral can be rendered nonneutral when the sensitivity coefficient of the enzyme increases as a result of the evolution of a different enzyme governing some other step along the metabolic pathway.

The repeated shifting and swaying of sensitivity coefficients resulting from natural selection implies that enzymes that are functionally linked in one or more metabolic pathways will evolve, in turn, in repeated cycles. Furthermore, metabolic pathways do not evolve in isolation, but are functionally linked in a manner analogous to enzymes within a single pathway. Consequently, as evolution improves the enzymes in a single pathway, the flux through the pathway will increase, until a point of diminishing returns is attained in which further increases in flux result in disproportionately smaller increases in fitness. Thus, the focus of selection ultimately shifts from improving the activity of individual enzymes to that of regulating and coordinating the various fluxes within the organism.

Enzymes with sharply defined optima of activity: The curve in Figure 1 must ultimately decline as the enzyme activity increases, as KACSER and BEEBY (1984) have pointed out, if for no more profound reason than that the cell or organism must eventually reach a point at which the cost of producing excess enzyme greatly outweighs the benefit in fitness that can be derived from possessing the excess. Thus, in this general sense, all enzymes have an optimal activity, and the key point is whether the optimum is very broad, which is the implication of Figure 1 as drawn, or very narrow. In many, perhaps most, cases the optimum must be very broad, as evidenced by the fact that the values

of a derived in Table 1 are very far out along the plateau. However, it is to be expected that, in some individual cases, the optimum will be very sharply defined, leaving little scope for changes in enzyme activity without decreasing fitness nearly proportionally. In such cases, a mutation could be selectively neutral only if the mutant enzyme were biochemically identical, or virtually identical, with the wild-type. In this manner, the existence of the sharp optimum constrains the acceptable types of variation in enzyme structure. LATTER (1975) has incorporated optimal enzyme activity in his interpretation of observed allozyme frequencies.

Multifunctional enzymes and pleiotropy: In this discussion we use the term "multifunctional" in a very wide sense to include enzymes or other gene products that are subject to two or more distinct selection pressures. Examples include enzymes that possess two catalytic sites for different substrates, as well as allosteric enzymes that interact with an inhibitor, such as commonly occurs in the feedback inhibition of the first enzyme in linear biosynthetic pathways, or with an activator. With multifunctional enzymes the fitness-activity relation depicted in Figure 1 becomes a surface in several dimensions, complicated further by the fact that the values along the axes of activity may be positively or negatively correlated, and an increased capability in one function may not be possible without some sacrifice in another function.

With a suitable definition of activity, the multidimensional fitness-activity surface may be projected into two dimensions, but the competing functions may result in a two-dimensional fitness-activity curve that has a very sharp optimum, or perhaps its shape will be very different than that depicted in Figure 1. Thus, competing functions can place evolutionary constraints upon enzymes and other gene products, and simple saturation theory may no longer be appropriate.

Pleiotropic genes affect two or more seemingly unrelated aspects of phenotype. A classical example is found in the sickle-cell β chain of hemoglobin, which functions to carry oxygen but also results in partial resistance to malaria (primarily heterozygotes) and severe anemia (homozygotes). The diverse phenotypic effects of a pleiotropic gene may be subject to conflicting selection pressures, and thus may result in a multidimensional fitness surface analogous to that discussed with multifunctional enzymes.

Although the hemoglobin molecule potentially affects every organ and tissue through the amount of oxygen that the molecule can supply, the hemoglobin molecule appears to offer wide scope for neutral or nearly neutral amino-acid substitutions to occur throughout the course of evolution. PERUTZ (1983) has noted that human and horse hemoglobin differ in sequence at 42 of 287 positions, yet their functional characteristics are indistinguishable. Indeed, among the approximately 400 known amino-acid substitutions in the hemoglobin molecule, only a minority affect any of the hemoglobin functions to a significant extent (PERUTZ 1983).

Branched pathways and other complications on metabolism: Although saturation kinetics appears to characterize many, and perhaps most, enzymes in intermediary metabolism (KACSER and BURNS 1973, 1979; SAVAGEAU 1976), there

are important exceptions. For example, KACSER (1983) discusses the paradoxical case of a negative sensitivity coefficient of a modulated enzyme in the arginine pathway in *N. crassa*, the result of which is that the flux of constituents proximal to the modulated enzyme (argininosuccinase) decreases as the activity of the modulated enzyme increases. However, KACSER argues that examples of this kind cannot be very numerous and, in most instances, probably would arise as artifacts of experimentally isolating a small part of a much larger system.

A second kind of complication in metabolism results from pathways in which one of the enzymes exhibits a sensitivity coefficient close to unity and is therefore "rate limiting." This situation seems to hold for many enzymes in biosynthetic pathways that are subject to feedback inhibition (SAVAGEAU 1975). Saturation theory does not apply to enzymes with high sensitivity coefficients, because in such cases the change in flux will be nearly proportional to change in activity. However, the metabolic theory of KACSER and BURNS (1973, 1979) implies that other enzymes in the same pathway must necessarily have sensitivity coefficients close to zero, and for these enzymes the saturation theory holds all the more strongly.

DISCUSSION

Many key aspects of the saturation theory discussed here have been touched upon by other authors in different contexts. The distinguishing feature of our approach is the assumption that natural selection will result in the occurrence of evolution along the trajectory of a concave fitness function. We infer from this assumption that natural selection will continue until an enzyme activity is achieved beyond which an additional increase in enzyme activity results in a negligible increase in fitness. At this point the fate of new mutations that have small effects on enzyme activity will be determined principally by the effects of random genetic drift. Thus, we deduce that evolution by means of the random genetic drift of neutral or nearly neutral mutations is not only consistent with the Darwinian theory of natural selection but also that the existence of nearly neutral alleles follows as a consequence of long continued natural selection of an enzyme gene. In this somewhat ironic sense, the greater the selectionist one might be, the greater the neutralist one should become.

Our deductions regarding the progress of enzyme evolution follow from the assumption of saturation kinetics, which has been shown to hold empirically in a number of examples (FLINT, PORTEOUS and KACSER 1980; KACSER and BURNS 1981; FLINT *et al.* 1981; MIDDLETON and KACSER 1983) and which is well supported on general theoretical grounds (KACSER and BURNS 1973, 1979; SAVAGEAU 1976; HEINRICH and RAPOPORT 1974, 1983). However, not all enzymes do follow such simple kinetics, and the principal complications can be classified under several headings—overdominance, fluctuations in the environment, enzymes with sharp optima, multifunctional enzymes, pleiotropic genes, and branched pathways. Thus, we do not propose that all enzymes undergo the same pattern of evolution as proposed for enzymes with saturation kinetics. However, we do propose that saturation kinetics is sufficiently well documented

and sufficiently widespread that the saturation theory of evolution can account for the observed characteristics of a substantial number of enzyme loci, particularly of housekeeping and other types of enzymes that have traditionally been included in allozyme surveys.

The saturation theory has great heuristic value when applied to the genes within its purview, and the theory provides many otherwise seemingly unrelated phenomena and observations with a common theme, including theories of the control of flux in metabolic pathways (KACSER and BURNS 1973, 1979), the neutral theory of molecular evolution (KIMURA 1968, 1983a, 1983b), the molecular basis of dominance (WRIGHT 1934; KACSER and BURNS 1981), the theory of concave fitness functions (GILLESPIE 1976, 1978), the hypothesis of slightly deleterious effects among mutations that are fixed by random genetic drift (OHTA 1976), observations on the heterozygous effects of null mutations and detrimental and lethal genes in *Drosophila* (MUKAI *et al.* 1972; OHNISHI 1977; LANGLEY *et al.* 1981), studies of the fitness and potential for selection among naturally occurring electrophoretic variants in *E. coli* (DYKHUIZEN and HARTL 1980, 1983; HARTL and DYKHUIZEN 1981, 1985) and observations of the fitness of laboratory mutations affecting β -galactosidase in *E. coli*.

The arguments for the saturation theory are most easily considered in the context of housekeeping enzymes, which also provide the best evidence for saturation kinetics; however, the theory may also apply to certain classes of regulatory genes or to genes that code for structural proteins. For example, genes that regulate the production of an enzyme may merely increase or decrease its amount and would, therefore, be subject to the imperatives of the saturation theory. However, a regulatory gene might also affect the timing of enzyme expression or the tissue specificity of expression, and in these situations the applicability of saturation theory is unclear. Likewise, it is very uncertain whether saturation theory in the simple form discussed here can be applied to the alleles of genes that regulate major developmental processes or that determine the fate of cell lineages. Certain aspects of the theory might be applicable to regulatory genes, but much more information about such genes is needed in order to decide the issue.

We have not touched on the important subject of the natural selection of quantitative traits, but the principles of saturation kinetics might profitably be considered in this context. The genotypic value of an individual with respect to a quantitative trait will often be determined by the flux across one or more metabolic pathways; for example, the pathways concerned with the production and utilization of growth hormone. In many cases, fitness may be greatest among individuals in whom the flux is intermediate. However, natural selection for an intermediate optimum value of flux is entirely compatible with low sensitivity coefficients for most enzymes in the relevant pathways, and thus, stabilizing selection of this type, like metabolism in general, will admit of many neutral or nearly neutral mutations. The possibility of extensive neutral evolution occurring under stabilizing selection has been inferred on independent grounds by KIMURA (1981), and considerations relevant to directional selection have been discussed by MILKMAN (1978) and CROW and KIMURA (1979).

Perhaps the most useful attribute of the saturation theory is the formulation of one type of Darwinian natural selection in terms of a simple biochemical model of enzyme action, but nevertheless, a model that appears to be valid at least to a first approximation in a wide variety of metabolic contexts. Thus, saturation theory may prove useful as a clear and simple backdrop, undoubtedly somewhat oversimplified, against which more complex real examples and exceptions may be projected.

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Note added in proof: A group of 12 distinguished contributors to metabolic control theory have recommended that sensitivity coefficients be renamed control coefficients (see BURNS, J. A. *et al.*, 1985 Control analysis of metabolic systems. Trends Biochem. Sci. **10**: 16).

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