DIRECTED EVOLUTION OF METABOLIC PATHWAYS IN MICROBIAL POPULATIONS. I. MODIFICATION OF THE ACID PHOSPHATASE pH OPTIMUM IN *S. CEREVISIAE*[1]

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ABSTRACT

An experimental system for directing the evolution of enzymes and metabolic pathways in microbial populations is proposed and an initial test of its power is provided.——The test involved an attempt to genetically enhance certain functional properties of the enzyme acid phosphatase in *S. cerevisiae* by constructing an environment in which the functional changes desired would be "adaptive". Naturally occurring mutations in a population of 10^9 cells were automatically and continuously screened, over 1,000 generations, for their effect on the efficiency (K_m) and activity of acid phosphatase at pH 6, and for their effect on the efficiency of orthophosphate metabolism.——The first adaptation observed, M1, was due to a single mutational event that effected a 30% increase in the efficiency of orthophosphate metabolism. The second, M2, effected an adaptive shift in the pH optimum of acid phosphatase and an increase in its activity over a wide range of pH values (an increment of 60% at pH 6). M2 was shown to result from a single mutational event in the region of the acid phosphatase structural gene. The third, M3, effected cell clumping, an adaptation to the culture apparatus that had no effect on phosphate metabolism.——The power of this system for directing the evolution of enzymes and of metabolic pathways is discussed in terms of the kinetic properties of the experimental system and in terms of the results obtained.

WE shall address ourselves to the structural and functional molecular mechanisms and processes that dictate the genetic and biochemical strategies of evolution. Some of these have been elucidated through studies of protein and nucleic acid structure and through studies of the kinetics of their short term interactions. Thus far the best understood molecular mechanisms and processes are conservative (e.g., information coding, information storage, information replication, replication error repair, etc.). On the other hand, the crucial features of these conservative mechanisms and processes (particularly the manner and frequency with which they break down and generate error) and other nonconservative molecular mechanisms responsible for observed biochemical strategies of genetic adaptation, have been less amenable to experimental analysis.

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One useful approach for elucidating the relevant genetic–biochemical mechanisms of the evolutionary process has been via comparative studies of the structure and function of homologous proteins in related organisms, e.g., hemoglobin (BUETTNER-JANUSCH and HILL 1965), cytochrome c (MARGOLIASH and SMITH 1965), tryptophane synthetase (BONNER, DE MOSE and MILLS 1965). The presumption here is that as the structural and functional properties of such homologs become well known, the molecular mechanisms and processes that contributed to their evolution will become more apparent (ZUCKERKANDL and PAULING 1965). It is a hindsight approach to the problem.

Analysis of the evolutionary process in motion would be a more direct means for searching out the genetic–biochemical parameters involved than inductive guesses based upon studies of the properties of its end products. The problem with this approach, however, is that an experimental system in which the evolution of proteins, metabolic pathways, regulatory mechanisms, etc., can be observed, has not yet been found. Such a system requires an experimental organism that can be maintained in large populations and that can be moved through significant periods of evolutionary time in relatively short periods of real time; i.e., an organism with a very short generation time. Furthermore, it requires a system in which the experimenter can select for mutations that increase the efficiency of specific metabolic pathways and, preferably, that increase specific functional capabilities of given enzymes in such pathways—contrary to the usual microbial systems that select only defective mutants.

We propose that certain microbial populations (including haploid, diploid, sexual and asexually reproducing microorganisms) maintained in chemostat environments provide an experimental system in which this can be done; and report here the results of our initial experiment designed to determine whether the evolution of metabolic pathways in microbial populations can be directed, in this system, and whether the relevant molecular interactions involved can be resolved. Our more specific objective was to elucidate the physical–biochemical basis of genetic adaptation of *Saccharomyces cerevisiae* to an environment in which its fitness was made highly dependent upon its rate and/or efficiency of phosphate metabolism.

THE EXPERIMENTAL SYSTEM

Kinetics of growth and reproduction in the chemostat: The basic kinetics of growth and reproduction of microbial populations in chemostats have been reviewed by MÁLEK and FENCL (1966). A chemostat is a continuous culture apparatus used to maintain microbial populations at static growth and reproductive rates under constant environmental conditions over long periods of time. Its unique features derive from the fact that at sufficiently low concentrations of any chosen essential nutrilite, growth and reproductive rates become dependent upon the rate of metabolism of that essential nutrilite. The relationship between the *specific reproductive rate* of the population, $\mu = \ln 2/(\text{population doubling time in hours})$, and the concentration of the essential nutrilite in short supply, s, has been shown empirically to approximate the relationship

$$\mu = \mu_{\max}\left[\frac{s}{K_s + s}\right];$$

where K_s is an empirical parameter analogous to the Michaelis-Menton constant of enzyme kinetics, and is defined as the concentration of the essential nutrilite in short supply that will sustain exactly half the maximum specific reproductive rate, $\mu_{\max}$, the rate that pertains when all essential nutrilites are plentiful.

This expression is merely an explicit statement of the usually observed asexual reproduction of microbial populations in flask cultures. When ample amounts of all essential nutrilites are available, microbial populations tend to reproduce at rates that result in an exponential increase in population density. This rate we denote as $\mu_{\max}$. As reproduction continues and cell density increases, all nutrilites drop in concentration at rates proportional to their rates of metabolism. If, for the sake of this discussion, we neglect the possible effects of accumulating metabolic poisons on reproductive rate, then the first essential nutrilite to fall in concentration to a value that limits its own rate of metabolism will limit reproductive rate. Without the addition of fresh medium both the concentration of the limiting essential nutrilite and the reproductive rate eventually drop to zero.

In a chemostat system, on the other hand, fresh medium is continuously added to the culture vessel at a rate, f, while spent culture medium and cells are washed out of an overflow at the same rate, resulting in a continuous dilution of the culture at a rate, $f/v = D$ (where v is the volume of the culture vessel). Thus, the culture medium is renewed and the cell population diluted at the same rate, D.

An initial cell population would enjoy an ample supply of limiting essential nutrilite in the culture vessel, and thus reproduce at the maximum rate, $\mu_{\max}$. If the dilution rate, D, were less than $\mu_{\max}$, population density would increase because cells would be produced faster than they would be washed out of the system. As the population grew, the amount of limiting essential nutrilite metabolized per unit time would increase and eventually surpass the rate, f, at which it was introduced to the culture vessel. This would result in a drop in its concentration to a value that would sustain a specific reproductive rate exactly equal to the dilution rate. At this point population density would be equilibrated because cells would be washed out at exactly the same rate that they were being produced. This equilibrium would remain *stable* with respect to the concentration of all components of the experimental system and with respect to the rates of all processes (including growth and reproduction) occurring therein. The chemostat is, thus, a "static rate biological reactor".

Features of genetic adaptation in the chemostat salient to this experiment: In a chemostat that has achieved its stable equilibrium, reproduction progresses at a constant rate—but always below that which would pertain if all nutrilites were plentiful. The controlling factor for this equilibrium is the low concentration of the single essential nutrilite that limits population reproductive rate. This creates an environment in which the "fitness" of cells is narrowly dependent upon the rate and/or efficiency with which they metabolize low concentrations of the critical nutrilite. Consequently, the effect of a new mutation on a cell's reproductive rate, and thus on genetic survival, depends upon how it affects the metabolism

of this essential nutrilite. A mutation that does not affect the metabolism of the critical nutrilite should have no effect on fitness, unless it depresses the rate of some other essential metabolic pathway to a point where it imposes the primary constraint on reproductive rate—in which case it depresses fitness and consequently would be washed out of the system.

On the other hand, a mutant that enhances the rate this crucial nutrilite is metabolized at, or that improves its metabolic efficiency, also enhances reproductive rate. Such a mutant, if it survived the contingencies of sampling in the first few generations, would increase in frequency at a rate dependent upon its increment in fitness and eventually replace the parent strain in the culture vessel. A new stable equilibrium would then be established in which the increment in "adaptation" of the population to the experimental environment would be reflected in an increased population density and an increased rate of biomass production (POWELL 1958).

The two features of this system that are of great relevance to population genetics and evolution derive from (1) the ease with which the experimenter *can automatically and continuously focus selection on given enzymes and metabolic pathways over significant periods of evolutionary time* (1,000 generations in this experiment) for (2) *mutations that enhance specific functional properties of such enzymes and the efficiency of such pathways*. Aside from the obvious use of such systems for modifying enzymes to maximize their functional capabilities under particular conditions to meet specialized requirements in biochemistry and medicine, e.g., the production of antibiotics (HOLME 1969) and of chemotherapeutic agents (LEVISOHN and SPIEGELMAN 1969), this system should also provide the evolutionist with a powerful tool for analyzing the molecular kinetics of genetic adaptation. The following experiment was designed to examine this possibility.

Selection was focused on the capability of acid phosphatase to catalyze the hydrolysis of the organic phosphate compound β-glycerophosphate and concomitantly on the efficiency with which liberated orthophosphate is metabolized. This was accomplished by constructing a chemostat environment with phosphate provided at a limiting concentration in organic form, β-glycerophosphate, and with the culture medium buffered at pH 6, a value that reduced acid phosphatase activity about 70%. Selection therefore favored mutations increasing the rate at which acid phosphatase catalyzed the hydrolysis of very low concentrations of β-glycerophosphate under the adverse pH condition pertaining, and also favored mutations increasing the efficiency of orthophosphate metabolism.

Naturally occurring mutants in a genetically "homogeneous" population of 10^9 individuals (derivatives of a single haploid cell by asexual reproduction) were screened in this environment for approximately 1,000 generations of asexual reproduction (approximately 8 months) and selected on the basis of their effect on reproductive rate. Adaptive mutants were analyzed to establish the genetic and physical–biochemical bases of their adaptations.

MATERIALS AND METHODS

Yeast strains: The haploid *Saccharomyces cerevisiae* strains S288C and XC500A, used in the adaptation experiment and in genetic tests, respectively, were provided by Dr. S. R. SNOW.

S288C is a strain of α mating type that carries three known mutations, gal_2, *mel*, *ma*, rendering the strain incapable of metabolizing galactose, melibiose and maltose. XC500A is a strain derived from S288C through autodiploidization and sporulation; its mating type is *a*. Strain MC1, resistant to canavanine, was isolated in our laboratory from S288C. The hapolid acp^- (acid phosphatase negative) strain, E1, of α mating type, used in genetic tests was also derived from S288C. It was provided by Dr. J. C. KUHN.

Acid phosphatase (E.C.3.1.3.2): This enzyme in *S. cerevisiae* is a nonspecific esterase localized in the cell wall (SCHMIDT *et al.* 1963), which catalyzes the hydrolysis of phosphate ester bonds; liberated orthophosphate is then transported into the cell by a permease system (ROTHSTEIN and DONOVAN 1963). Estimates of the enzyme pH optimum lie between 3 and 4 (BOER and STEYN-PARVE 1966), within which range it is capable of supplying phosphate at a rate that is in excess of the cell's requirement. Using acp^- mutants KUHN (1969) showed that acid phosphatase in *S. cerevisiae* is coded by a single structural gene.

The constraints of the experimental environment effectively focused selection on the efficiency (K_m) and the activity of this enzyme. One constraint was achieved by providing phosphate solely in organic form as β-glycerophosphate. KUHN (1969) showed that mutants unable to make normal acid phosphatase (acp^- mutants) were unable to utilize exogenously supplied β-glycerophosphate as a phosphate source. The second constraint was imposed by buffering the culture medium at pH 6. In agreement with estimates of BOER and STEYN-PARVE (1966) and others, that acid phosphatase activity is sharply reduced at pH 6, we estimated a reduction of about 70%. This reduction resulted in a 25% reduction in maximum specific reproductive rate, μ_{max}, on β-glycerophosphate, (from 0.305 to 0.227) a reduction not evident at pH 6 with orthophosphate as phosphate source nor at pH 4 regardless of phosphate source.

Media: The amounts of the various ingredients indicated are those required for the preparation of one liter of medium. 2% agar was added for solid medium. (1) Minimal: Difco yeast nitrogen base without amino acids; dextrose, 20 g. (2) Buffered minimal: ¼ strength minimal medium (to minimize contaminant phosphate ion concentration) with KNO_3 substituted for KH_2PO_4; Tris(hydroxymethyl) aminomethane, 6.05 g, maleic acid, 5.8 g, NaOH, 1.2 g (a pH 6, 0.05 M buffer); filter-sterilized β-glycerophosphate (disodium salt, pentahydrate) (βGP)* added to concentrations specified below. (3) Yeast extract peptone (YEP): yeast extract, 10 g; bactopeptone, 20 g; dextrose, 20 g. (4) Presporulation: yeast extract, 2.5 g; nutrient broth (Difco), 8.0 g; dextrose, 100 g. (5) Sporulation: yeast extract, 2.5 g; potassium acetate, 9.8 g; dextrose, 1 g. (6) Canavanine selective: minimal medium with 60μg/ml canavanine.

Crosses: Strains of opposite mating type were grown overnight on YEP agar. Small amounts of cells from both strains were mixed on YEP agar. After three hours zygotes had formed and several of these were isolated by micromanipulation.

Sporulation and ascus dissection: Diploids to be sporulated were inoculated into presporulation medium and growth allowed to proceed to the stationary phase (about three days) on a shaker at 30°C. This culture was harvested by centrifugation, washed with water and samples transferred to solid sporulation medium and incubated at 30°C. When sporulation had occurred (within one week), asci were suspended in snail digestive juice to soften the ascus wall. Spores of four-spored asci were isolated by micromanipulation (JOHNSTON and MORTIMER 1959).

Specific reproductive rate of mutants in the experimental environment, $\mu^i{}_{s_0}$: At the concentration of essential nutrilite pertaining in an equilibrium chemostat, adaptive mutants reproduce at a greater rate than the strain from which they arise; i.e., at a rate greater than the dilution rate, D. The increment in reproductive rate provides a measure of the increment in "fitness" conferred by the mutation. We estimated the specific reproductive rates of the n adaptive mutants at the concentration, s_o, of essential nutrilite pertaining in the initial equilibrium population of S288C reproducing at the rate 0.151, and denote estimates of the specific reproductive rate of the i_{th} mutant obtained under these conditions as

$$\mu^i{}_{s_0} = \mu^i{}_{max} \left[\frac{s_0}{K^i{}_{s_0} + s_0} \right] . \qquad (1)$$

* The abbreviation βGP will be used for β-glycerophosphate.

This was done by reconstructing the initial equilibrium chemostat, inoculating the adaptive strain into this equilibrated chemostat and assaying the rate at which the adaptive strain displaced the resident strain. In order to distinguish the adaptive strain from the resident strain in these assays, a canavanine-resistant derivative of S288C (denoted as MC1) was utilized as the resident strain. (It was established with 95% confidence that, under the conditions of these experiments, if the MC1 and S288C strains do differ in specific reproductive rate, it is by no more than $\pm$ 0.0012.)

We estimated $\mu^{i}{}_{s_0}$ by assuming displacement of MC1 by the adaptive mutant proceeded in the chemostat according to the equation

$$p_t = p_0 e^{-(\mu^{i}{}_{s_0} - D)t}$$

where p_t is the frequency of MC1 cells in the population at time t, p_0 is the frequency of MC1 cells in the initial equilibrium population, and $(\mu^{i}{}_{s_0} - D)$ is the difference in reproductive rate between the two competing strains. This equation is strictly valid only if there is no interaction between the strains. Also it does not account for the fact that $\mu^{i}{}_{s_0}$ eventually recedes to D as a result of the reduction in the concentration of limiting nutrilite as population density increases to its new equilibrium value. This simplication, however, apparently did not introduce a serious bias in our estimates of $\mu^{i}{}_{s_0}$ since plots of $(\ln(\hat{p}_t)/\text{time})$ were linear in all experiments.

MC1 frequency at time t, p_t, was estimated by plating a population sample taken at time t on solid canavanine selective medium (a medium which will support growth of MC1 only), counting those clones that arose after four days incubation at 30°C and dividing this count by the total number of cells in the original sample. (All cell counts were made with a Celloscope III, Particle Data, Inc., Elmhurst, Ill.).

Sampling in this instance was analogous to taking a random sample, $x_1, x_2, \ldots, x_n$, of size n from the point binomial density

$$f(x) = p^x{}_t(1 - p_t)^{1-x} \qquad x = 0, 1; \qquad 0 \leq p_t \leq 1,$$

a density with mean p_t and variance $p_t(1 - p_t) = p_t q_t$. An estimate of the mean p_t is provided by the maximum likelihood estimator $p_t = 1/n \Sigma x_i$. From a consideration of the central-limit theorem it is evident that the estimator p_t is approximately distributed as a normal variate with mean p_t and variance $p_t q_t/n$, and that the probability statement

$$P(|\hat{p}_t - p_t| < 0.01) = 0.95$$

will be true for any values of p_t and q_t if the sample size is always greater than or equal to 9,604—a sample size well within the technical limits of the experimental system.

The estimator $\ln(p_t)$ is also a maximum likelihood estimator and is normally distributed. To obtain estimates of $\mu^{i}{}_{s_0}$ the regression of $\ln(\hat{p})/\text{time}$ was employed according to the equation

$$\ln(p) = \alpha + \beta t + e$$

where $\beta = (\mu^{i}{}_{s_0} - D)$. Maximum likelihood estimators exist for α, β, and σ^2 (Mood and Graybill 1963).

With an estimate of β and knowing the value of D (the dilution rate, 0.151, at which all estimates were made), an estimate of $\mu^{i}{}_{s_0}$ is readily obtained.

$\mu^{i}{}_{max}$ estimation: In an operating chemostat, the i_{th} mutant population, if its density is sufficiently below the equilibrium density, will reproduce at its maximum rate, $\mu^{i}{}_{\max}$, because all essential nutrilites are in excess, and will increase in density according to the equation

$$N_t = N_0 e^{(\mu^{i}{}_{\max} - D)t}$$

where N_t is the density of the population at time t, N_0 is the density initially, and $(\mu^{i}{}_{\max} - D)$ is the difference between the maximum reproductive rate of the ith mutant and the rate at which the culture is being diluted. With a high dilution rate several days (20–30 generations) may be required before density increases to the point that the concentration of an essential nutrilite becomes limiting. Estimates of $(\mu^{i}{}_{\max} - D)$ were taken from the regression of $\ln(N_t)/\text{time}$ according to the equation

$$\ln(N) = \alpha + \beta t + e$$

where $\beta = (\mu^{i}{}_{\max} - D)$. ($D = 0.010$ in all experiments.)

Chemostat culture conditions: All cultures were maintained at 30°C. (1) Adaptation experiment: buffered minimal medium with 5.0 μg/ml βGP; culture volume, 191 ml; flow rate, 23 ml/hr. (2) $\mu^{i}{}_{s_0}$ estimation: buffered minimal medium with 15.0 μg/ml βGP; culture volume, 186 ml; flow rate 28 ml/hr. (3) $\mu^{i}{}_{\max}$ estimation: buffered minimal medium with 10 g/l dextrose to eliminate the possibility of dextrose limitation; culture volume, 385 ml; flow rate, 38 ml/hr. The estimate of $\mu^{i}{}_{\max}$ in an organic phosphate environment was conducted with 100.0 μg/ml βGP. The estimate of $\mu^{i}{}_{\max}$ in an inorganic phosphate environment was conducted with 1.0 g/l KH_2PO_4. (4) Estimation of enzyme activity, K_m, pH optimum, yield constant and equilibrium substrate concentration: Chemostat operating conditions were identical to those outlined above for $\mu^{i}{}_{s_0}$ estimation.

Phosphate determination: The method of MURPHY and RILEY (1962) with modification was used for orthophosphate determination. (It is a highly sensitive method giving an optical density of 1.00 with 26.9 μg P per 25 ml.) To 20.0 ml H_2O were added 1.0 ml of solution whose phosphate concentration was to be determined and 4.0 ml of the phosphate reagent of MURPHY and RILEY. The color produced was stable for six hours and was read spectrophotometrically at 720 mμ. This method is based on reduction of an ammonium molybdiphosphate complex by ascorbic acid in the presence of antimony. STEYN-PARVE (1962) found that molybdate has a powerful inhibitory effect on acid phosphatase, sufficiently powerful to completely inhibit acid phosphatase activity in the *in vivo* assays discussed here.

β-glycerophosphate assay: Chemostat effluent samples, with cells removed by vacuum filtration, were treated initially with concentrated HNO_3 to hydrolyze βGP; excess carbon was then removed by treatment with 10% $Mg(NO_3)_2$, the residue dissolved in 1.0N HCl, and orthophosphate concentration determined.

$K^{i}{}_{s_i}$ *estimation:* An estimate of $K^{i}{}_{s_i}$ is provided by solution of equation (1) using estimates of $\mu^{i}{}_{\max}$ and s_i (the concentration of βGP pertaining in an equilibrium chemostat population of the i_{th} mutant), and the relationship $\mu^{i}{}_{s_i} = D$. This parameter is a measure of the efficiency with which the i_{th} mutant population is able to metabolize the limiting nutrilite (βGP) and reproduce in the experimental environment.

Yield constant estimation: Y^i = (equilibrium density of the i_{th} mutant population)/(βGP concentration required to maintain that equilibrium density); the required concentration is the difference between the concentration pumped into the system, s_r, and the effluent concentration, s_i. Yield is a measure of the efficiency of orthophosphate metabolism.

Enzymatic parameters (below) were estimated *in vivo*. (The equivalence of *in vivo* and *in vitro* acid phosphatase assays in *S. cerevisiae* has been confirmed by several authors, e.g., SCHMIDT *et al.* (1963), BOER and STEYN-PARVE (1966), KUHN (1969).) Chemostat effluent cells were collected for 3.5 hr under vacuum on a Millipore (0.45 mμ) membrane. All assays were conducted at 30°C and at pH 6 in order to establish adaptive enzymatic changes pertaining to the experimental environment. Accuracy was enhanced by removing cells (centrifugation) prior to spectrophotometric analysis.

Enzyme activity estimation: To a given volume, x, of 1.5×10^{-2} M βGP in pH 6, 0.05 M Tris-maleate buffer was added a volume, $x/2$, of cell suspension at a concentration of about 10^8 cells/ml in the above buffer. Samples were taken from the reaction mixture over a period of 16 min and orthophosphate concentration determined.

Enzyme pH optimum estimation: Eleven solutions of 1.5×10^{-2} M βGP in 0.1 M acetate buffer ranging in pH from 3.6 to 5.6 in increments of 0.2 were employed in enzyme activity assays as described above.

Enzyme K_m *estimation:* To aliquots (20.0 ml) of βGP ranging in molarity from 5×10^{-6} to 3×10^{-5} in 0.05 M, pH 6 Tris-maleate buffer was added 1.0 ml of cell suspension at a concentration of about 10^7 cells/ml in the above buffer. The reaction was terminated at 30 sec by the addition of the phosphate reagent of MURPHY and RILEY (4.0 ml). Data were analyzed according to the Lineweaver-Burk procedure. (Apparently K_m values estimated at pH 6 cannot be compared with estimates made at optimum pH. They are intended only for comparisons among the strains used in these experiments.)

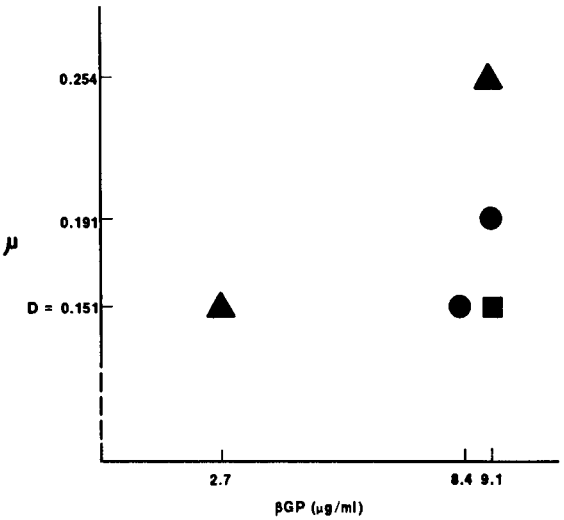

FIGURE 1.—Plot of the relationship between the specific reproductive rate, μ, and βGP concentration for S288C, ■; M1, ●; and M2, ▲.

RESULTS

Three transitions to higher states of adaptation to the experimental environment were observed. Two of these transitions resulted from adaptive changes in phosphate metabolism and led to new higher equilibrium population densities and, consequently, higher rates of biomass production. The third transition entailed an adaptation to the experimental apparatus and had no effect on phosphate metabolism nor on reproductive rate. Estimates of $\mu^{i}{}_{s_o}$, $\mu^{i}{}_{\max}$, $K^{i}{}_{s_i}$, and s_i for S288C and the i^{th} putative mutant are reported in Table 1.

The first adaptive event, resulting from a putative mutation, denoted as M1, was detected after about 180 generations. It effected a 25% increase in specific reproductive rate, $\mu^{1}{}_{s_o}$, at the concentration of βGP, s_o, pertaining when it arose (Table 1 and Figure 1), and a 30% increase in yield constant in the phosphate limiting environment, Y^1, a measure of the efficiency of orthophosphate metabolism (Table 2). (The yield increment is not maintained in phosphate-saturated

TABLE 1

Estimates of $\mu^{i}{}_{s_o}$, $\mu^{i}{}_{max}$, $K^{i}{}_{s_i}$ *and* s_i*

Strain	$\mu^{i}{}_{s_o}$	$\mu^{i}{}_{\max}$	$K^{i}{}_{s_i}$	s_i (μg/ml)
S288C and MC1	0.151†	0.227 ± 0.007	4.58	9.10 ± 0.18
M1	0.191 ± 0.001	0.230 ± 0.004	4.41	8.43 ± 0.16
M2	0.254 ± 0.006	0.261 ± 0.003	1.98	2.72 ± 0.30
M3	0.249 ± 0.003			

* Estimates are reported with 95% confidence intervals where possible.
† 0.151 is the dilution rate and specific reproductive rate of S288C and MC1 in a chemostat with an equilibrium βGP concentration s_o.

TABLE 2

Estimates of yield constant, Y

Strain	Population density (cells/ml) × 10^6	Utilized nutrilite (μg βGP/ml)	Y^4 (cells/μg βGP) × 10^5
S288C	4.1 ± 0.1*	5.90 ± 0.18	6.9
M1	6.0 ± 0.2	6.57 ± 0.16	9.1
M2	11.0 ± 0.2	12.28 ± 0.30	9.0

* Estimates are reported with 95% confidence intervals.

environments where maximum growth rates pertain and yield values are approximately six fold smaller.) It had no effect on acid phosphatase pH optimum, activity or K_m (Tables 3 and 4). M1 was crossed to strain XC500A to test the hypothesis that the adaptation had a genetic basis. Segregation for yield constant (9.1×10^5 cells/μg βGP : 6.9×10.5 cells/μg βGP) was 2:2 in each of four asci observed. No further genetic tests were undertaken with M1 because yield mutants that effected metabolic efficiency of orthophosphate were only of passing interest in this study.

The second adaptive event, resulting from a putative mutation, denoted as M2, was detected at about generation 400. The estimate of its specific reproductive rate, $\mu^2_{s_0}$, at the concentration of βGP, s_0, pertaining at the outset of the experiment is not significantly less than the estimate of its maximum reproductive rate in the experimental environment, μ^2_{max} (Table 1 and Figure 1). This indicates that the reproductive rate of M2 was not limited by the concentration of βGP, s_0, that pertained at the outset of the experiment, a concentration that limited the reproductive rate of S288C to 60% of its maximum of pH 6. The estimate of μ^2_{max} indicates that M2 has recovered about 40% of the reduction (from 0.305 to 0.227) in reproductive rate caused by buffering the culture medium at pH 6.

Results listed in Table 3 indicate M2 has effected a 60% increase in acid phosphatase activity at pH 6. Two things are apparent from the data listed in Table 4 and depicted in Figure 2: (1) the activity of M2 acid phosphatase is greater than that of S288C over the entire range of pH measured; and (2) the pH optimum has shifted from 4.2 to 4.8.

M2 was assayed for *in vivo* phosphatase activity at pH 8, the pH optimum of

TABLE 3

Estimates of acid phosphatase K_m *and activity at pH 6 with βGP as substrate**

Strain	K_m (M × 10^{-5})	Activity (μmole/min/cell) × 10^{-10}
S288C	$1.25 < 1.54 < 2.00$	6.72 ± 0.19
M1	$1.01 < 1.25 < 1.64$	6.51 ± 0.21
M2	$0.99 < 1.33 < 2.04$	10.65 ± 0.27

* Estimates are reported with 95% confidence intervals. The asymetric intervals are reciprocals of those estimated for the statistic (Lineweaver-Burk) $1/K_m$.

TABLE 4

Acid phosphatase activity estimates over a range of pH with βGP as substrate

pH	Activity* (μmole/min/cell) × 10^{-10} S288C	M1	M2
3.6	22.86 ± 0.16	23.07 ± 0.12	27.62 ± 0.13
3.8	22.85 ± 0.15	23.01 ± 0.10	28.23 ± 0.10
4.0	23.72 ± 0.11	23.48 ± 0.15	30.37 ± 0.17
4.2	25.84 ± 0.09	26.03 ± 0.12	31.86 ± 0.16
4.4	24.03 ± 0.25	23.78 ± 0.14	37.21 ± 0.11
4.6	22.53 ± 0.10	22.56 ± 0.14	40.23 ± 0.17
4.8	21.62 ± 0.18	21.25 ± 0.15	42.69 ± 0.16
5.0	19.84 ± 0.12	19.65 ± 0.15	34.20 ± 0.14
5.2	16.87 ± 0.10	16.98 ± 0.16	26.75 ± 0.12
5.4	11.43 ± 0.13	11.49 ± 0.09	20.48 ± 0.15
5.6	8.09 ± 0.08	8.41 ± 0.18	15.62 ± 0.09
6.0†	6.72 ± 0.19	6.51 ± 0.21	10.65 ± 0.27

* Estimates are reported with 95% confidence intervals.
† Estimates at pH 6 were made with a different buffer; see MATERIALS AND METHODS.

alkaline phosphatase, to test the possibility that its increased acid phosphatase activity was due to leakage of alkaline phosphatase from the cell. (Apparently alkaline phosphatase is the only other phosphatase of *S. cerevisiae* capable of catalyzing the hydrolysis of βGP (KUHN 1969.) No activity was detected.

M2 was crossed to XC500A to determine whether its increased enzyme activity

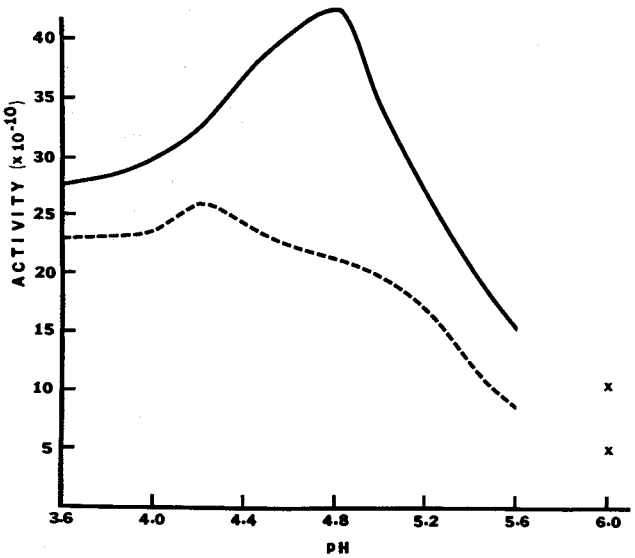

FIGURE 2.—Acid phosphatase activity over a range of pH with βGP as substrate. The solid line was drawn through 11 estimated activities of M2 listed in Table 4. Similarly, the broken line was drawn through the average activities, at each of the 11 pH values, of S288C and M1. Estimates at pH 6, denoted by X, were made with a different buffer.

and pH optimum shift had a genetic basis. Fifteen asci were analyzed for pH optimum segregation (4.2 : 4.8). Segregation was 2:2 in all asci. Three segregants from this cross, with 4.8 pH optimum and *a* mating type were crossed with the acp^- strain, E1. Six asci were analyzed from each of these three crosses for the segregation of acp^-:acp^+ with 4.8 pH optimum. All eighteen asci analyzed segregated 2:2 in this respect. No wild-type (pH 4.2) segregants were recovered. These results indicate that the increased activity and pH optimum shift in M2 acid phosphatase are the results of a single mutational event in the region of the acid phosphatase structural gene of C288C.

A third adaptive event, resulting from a putative mutation, denoted as M3 and characterized by cell clumping, was detected at about generation 800. Clumps have a slightly greater settling rate than free cells. Thus, despite the quite vigorous stirring action of an air sparge in the culture vessel, the probability of clumpers being washed out of the *overflow* was apparently slightly less than the washout probability of free cells. The technique of using an overflow to maintain constant culture volume would be expected to confer a slight competitive survival advantage on clumpers. The technique utilized to estimate $\mu^{i}{}_{s_0}$ (MATERIALS AND METHODS) does not discriminate between the effects of differential survival and differential reproductive rates on changes in gene frequency. Thus, the increase in the clumpers' survival probability should be reflected in an increase in the estimate of specific reproductive rate at the βGP concentration, s_0. No significant difference between $\mu^{2}{}_{s_0}$ and $\mu^{3}{}_{s_0}$ was detected, however, indicating that the increment in survival probability of clumpers must be quite small. No increase in population density was observed indicating that M3 had no significant effect on phosphate metabolism. It was concluded that M3 represents an adaptation to the experimental apparatus rather than to the constraints of the experimental environment. Thus, it was of no interest here and was therefore analyzed no further.

The three known mutations in S288C, gal_2, *mel*, *ma.* were utilized to determine whether the adaptation experiment concluded with a variant strain of S288C or with some contaminant. Strain S288C and chemostat effluent samples taken just prior to conclusion of the adaptation experiment were inoculated into separate flasks containing one of the carbohydrates, dextrose, galactose, melibose or maltose, as energy source. Growth was detected in all flasks supplied with dextrose while no growth was detected in the other flasks, indicating that the strain concluding the adaptation experiment carried the three S288C mutations. Crosses were attempted between the above effluent samples and the haploid strain of *a* mating type, XC500A, to further determine whether the population concluding the adaptation experiment was both haploid and of α mating type. The resulting putative zygotes were sporulated (four-spored asci), indicating that the concluding strain was both haploid and of α mating type as was the initial strain, S288C.

DISCUSSION AND CONCLUSIONS

We have proposed that certain microbial populations maintained in chemostat

environments provide an experimental system in which the evolution of metabolic pathways can be directed and the relevant molecular parameters involved can be resolved. Two criteria are utilized to assess the validity of this proposition: (1) the capability of the system for sustaining large, actively reproducing microbial populations under controlled conditions for significant periods of evolutionary time; and (2) the precision with which selection for enhanced efficiency of specific metabolic pathways and enhanced functioning of given enzymes, under a variety of conditions, can automatically and continuously be applied in this system.

The capability of the chemostat system for sustaining large, actively reproducing microbial populations under controlled conditions for a large number of generations has already been well documented. A wide variety of bacteria, fungi and algae (haploid and diploid, reproducing both sexually and asexually) have been cultured in chemostats (MÁLEK *et al.* 1969). In fact, stable equilibria involving phage–bacterial populations (MÁLEK *et al.* 1969), two bacterial genera (POWELL 1958), and intra specific but genetically heterogeneous yeast populations (WASE and HOUGH 1965) have been maintained in chemostats. Thus, the maintenance of 10^9 cells through 1,000 generations, accomplished here, represents no special technical feat. The amount of evolutionary time that can be compressed, in this system, into a reasonable length of real time, however, is difficult to assess because there exist only very crude estimates of the per cell per generation probabilities of non-conservative genetic events that are evolutionarily significant. In this experiment 30% increment in the efficiency of orthophosphate metabolism and a 60% solution to the problem of low acid phosphatase activity at pH 6 were attained within the first 400 generations (10^9 cells $\times$ 400 generations $= 4 \times 10^{11}$ cell generations). No further progress, however, in alleviating the effect of pH on acid phosphatase was made in the remaining 600 generations (6×10^{11} cell generations). The rather trite partial solution to the pH problem (a single mutational event) is not evidence that it is a commonly occurring event at the acid phosphatase locus. If the mutation rate at the acid phosphatase locus is assumed to be about 10^{-5}, then approximately 4 million mutations at this locus were screened before a successful mutation arose; i.e., one that both incremented fitness (by about 30% in this case) and that survived the risk of loss due to sampling that exists in the first few generations when its frequency is extremely low. The lack of further progress in incrementing acid phosphatase activity at pH 6 indicates that a complete genetic solution to this problem is even rarer. In any case, it seems clear that where solutions of very low total probability are required (probabilities of less than 10^{-12}) either necessitating several sequential steps of low probability, or single events of very low probability (as might be necessary, for example, in the development of a new enzyme function), many more cell generations would be required for the experimenter to observe genetically and biochemically significant portions of the process. How many more is unclear.

Thus, from our results we can only conclude that the system we utilized is probably sufficiently powerful to allow studies of solution mechanisms for "sim-

ple" adaptation problems (those with per cell per generation probabilities equal to or greater than 10^{-12}) and possibly the first steps to solutions of adaptive problems that have a lower total probability. It does seem evident, however, that the power of this system to compress evolutionary time into real time could be increased easily by a factor of 10^3 without manipulating mutation rates that presumably might lead to still further increases in power by a factor of 10 or 10^2. This could be done by utilizing bacteria as an experimental organism. Bacteria can be maintained at at least one hundred times greater density than yeast, in this system, and they have significantly greater reproductive rates. This ploy should allow studies of solution mechanisms for adaptive problems that have total per cell per generation probabilities equal to or greater than 10^{-15}.

With respect to the second criterion, it seems clear that the degree to which selection for genetic events that result in enhanced performance can be focused, in this system, depends upon the degree to which the experimenter can define, delimit, and control the rate of specific metabolic processes essential to normal growth and reproduction. This is done in the chemostat by manipulation of the environment and/or the organism. In the experiment reported here the environment was manipulated to focus selection on genetic events that incremented the rate at which acid phosphatase hydrolyzed very low concentrations of β-glycerophosphate at pH 6, and that incremented the efficiency of orthophosphate metabolism. The fact that mutants were obtained that increased the activity of acid phosphatase at pH 6 and increased the efficiency of orthophosphate metabolism is evidence that the particular manipulation utilized had the expected effect. The results of HORIUCHI, TOMIZAWA and NOVICK (1962) provide additional evidence of the precision with which selection can be focused in the chemostat on mutations that enhance enzyme activity. With lactose-limited chemostat cultures of *E. coli*, they obtained genetically effected increments in the specific activity of β-galactosidase (from 0.07 to 3.5 units) within a thousand generations (apparently within about 2×10^{13} cell generations). The genetic mechanisms involved appeared to be a constitutive mutation in the *lac* operon followed by other mutational events in the *lac* region leading to the synthesis of β-galactosidase at hyper rates. They concluded that the hyper phenotype was either the result of a higher rate of operon transcription or the result of duplication within the *lac* region.

On the basis of these results and on the basis of known kinetic properties of chemostat systems (POWELL 1958) it seems evident that selection for further increments in the activity of acid phosphatase (at pH 6 or any other pH), or for activity with respect to unusual substrates, etc., could easily be affected. For example, the introduction of a competitive inhibitor of acid phosphatase to the environment of our current experiment could be utilized to reduce the effective activity of acid phosphatase to a value that would again focus selection on mutations that further increased acid phosphatase activity at any pH within its functional range. On these same bases it seems evident that by similar environmental manipulations selection could be focused on the efficiency of any other essential nutrilite pathway and on the activity, under a wide variety of conditions, of the external enzymes that provide the initial steps in such pathways.

Consequently, we conclude that selection in this system can be automatically and continuously focused to enhance the efficiency of at least some metabolic pathways and to enhance several specific functional properties of at least some enzymes in such pathways. Focusing selection on a specific functional property of an intermediate enzyme in such a pathway, however, would probably be more difficult and might require some sort of genetic manipulation of the organism—for example, effecting a deletion in the step preceding the enzyme of interest and limiting growth and reproductive rate by providing the organism with only a very low concentration of the product, or product analogue, of the missing enzyme. The effectiveness of such techniques in focusing selection in a chemostat over long periods of time, however, is as yet untested.

Finally, we conclude that the results obtained suggest some of the potential of this new experimental approach for elucidating the genetic–biochemical parameters of genetic adaptation in a wide variety of microorganisms including haploid, diploid, sexual and asexually reproducing species. Information of this sort should serve two purposes: (1) it should contribute to the construction of a physical–biochemical interpretation of some of the central dogma of population genetic theory, and lead toward an elucidation of genetic adaptation as a natural consequence of physical–biochemical law, thus integrating the disciplines of population genetics and evolution with those of biochemistry and molecular genetics; and (2) in a fashion analogous to the development of widely known and highly successful agricultural applications of population genetic theory, it should provide a basis for the development of theory, methodology and technology useful in directing the evolution of given microbial populations to optimize their rate and efficiency of substrate utilization while maximizing their rate of production of desired metabolic products.

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