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Continuous Culture of *Rhodotorula rubra*: Kinetics of Phosphate-Arsenate Uptake, Inhibition, and Phosphate-Limited Growth

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The pink yeast *Rhodotorula rubra* of marine origin was found to be capable of extended growth at very low phosphate concentrations ($K_{0.5}$ = 10.8 nM). Average intracellular phosphate concentrations, based on isotope exchange techniques, were 15 to 200 mM, giving concentration gradients across the cell envelope of about 10^6. Sensitivity to metabolic inhibitors occurred at micromolar concentrations. Inability of the phosphate transport system, K_s = 0.5 to 2.8 μM, V_{max} = 55 μmoles per g of cells per min, to discriminate against arsenate transport led to arsenate toxicity at 1 to 10 nM, whereas environmental arsenate levels are reportedly much higher. Phosphate competitively prevented arsenate toxicity. The K_i for phosphate inhibition of arsenate uptake was 0.7 to 1.2 μM. Phosphate uptake experiments showed that maximal growth rates could be achieved with approximately 4% of the total phosphate-arsenate transport system. Organisms adapted to a range both of concentration of NaCl and of *p*H. Maximal affinity for phosphate occurred at *p*H 4 and at low concentrations of NaCl; however, V_{max} for phosphate transport was little affected. Maximal specific growth rates on minimal medium were consistent in batch culture but gradually increased to the much higher rates found with yeast extract media when the population was subjected to long-term continuous culture with gradually increasing dilution rates. Phosphate initial uptake rates that were in agreement with the steady-state flux in continuous culture were obtained by using organisms and medium directly from continuous culture. This procedure resulted in rates about 500 times greater than one in which harvested batch-grown cells were used. Discrepancies between values found and those reported in the literature for other organisms were even larger. Growth could not be sustained below a threshold phosphate concentration of 3.4 nM. Such thresholds are explained in terms of a system where growth rate is set by intracellular nutrient concentrations. Threshold concentrations occur in response to nutrient sinks not related to growth, such as efflux and endogenous metabolism. Equations are presented for evaluation of growth rate-limiting substrate concentrations in the presence of background substrate and for evaluating low inhibitor concentration inhibition mechanisms by substrate prevention of inhibitor flux.

Although phosphate is a major nutrient and kinetic studies of nutrient-limited aquatic microbial systems are fairly common, few data exist specifying limiting phosphate concentrations at steady state. This is largely due to the fact that phosphate is a usual contaminant of chemicals and glassware, so that experimental systems with low and accurately known phosphate concentrations are difficult to prepare.

We first became interested in phosphate-limited systems after noting that dilute continuous culture was easier to start after adding large quantities of phosphate, and that phosphate seemed to prevent copper sensitivity in media low in trace metals (11), a phenomenon not predicted by usual binding constants (5). Phosphate-limited growth was studied with a pink yeast of marine origin (9). Although the

prevention of copper sensitivity in the low trace metal medium used was found to be due to contaminating manganese in the phosphate, the phosphate uptake kinetics themselves appeared interesting.

Discrepancies noted between the kinetics of phosphate flux into organisms during continuous culture and the initial uptake rate measured by methods usually employed (4, 16, 31) prompted development of alternative procedures employing continuous culture samples directly for flux measurements. The resulting transport rates measured were high, the attendant concentration gradients were large, and the systems were quite sensitive to metabolic inhibitors at low concentration.

Phosphate-arsenate interactions noted elsewhere (31, 33) were seen; however, mutual inhibition occurred at much lower concentrations, and levels were equivalent to those found in natural systems (22, 24). The kinetics of phosphate uptake, growth limitation, and inhibition of arsenate transport are described in view of their relevance to microbial processes in dilute aquatic environments.

MATERIALS AND METHODS

Organism. *Rhodotorula rubra*, a pink yeast recently isolated from seawater (9), selected for its ability to grow in continuous culture systems at low *p*H at the low phosphate concentrations employed, was used in these experiments. Identification of this strict aerobe according to Lodder (27) was *R. rubra* (Demme) in agreement with L. R. de Miranda (*personal communication*).

Continuous culture. Apparatus and general methods have been described (10). The medium employed was a dilute chelate-free mineral salts mixture (9), which was modified to contain low phosphate concentrations and which included vitamins B_{12}, thiamine, and biotin at 100 pM. The sodium chloride medium contained, in addition, 350 mM NaCl. The *p*H was 4.0 after autoclaving. Where specified, the *p*H was carefully adjusted to 7.0 with dilute NaOH after addition of 5 mM tris(hydroxymethyl)aminomethane buffer. Temperature was maintained at 25 C. Reservoirs of medium were agitated briefly after autoclaving to equilibrate with air. No additional oxygen was provided, thereby reducing the opportunity for gas-phase chemical contamination. Nutrient salts were of common origin, added from a single pair of compounded dry salt mixtures. When extracellular steady-state limiting nutrient concentrations were measured, organisms were removed by filtration. Samples were taken directly from the reactor with a syringe fitted with a filter and a two-way valve for rapid separation of the organisms from the sample. Undesirable perturbations in dilution rate during sampling were minimized either by appropriate feed rate adjustment after sampling or by replacing the removed sample with filtered air and then refilling the reactor at the operational dilution rate. Sample volumes were normally 15 to 20 ml from a 250- or 500-ml reactor. Feed reservoirs were 20-liter carboys fitted for in-place replenishment of expired medium (12). At least six volume changes were allowed for the steady state to establish. Population density averages were determined from data obtained by routine plate count and with a model B Coulter counter. Cell mass was determined from size distributions and microbalance data as previously described (12).

Phosphate uptake in continuous culture. Measurements and calculations are based on the usual relationships describing continuous culture. Although the basic relationships are available elsewhere (10, 12, 19, 32), those concepts particularly pertinent to the following treatment are reviewed for clarity. The reactor can be classified as stirred, single-stage, single-phase, continuous flow without recycling. Relationships assume perfect mixing, total population viability, and growth rate depending on the limiting substrate alone. Dilution rate is operationally set by supplying a reactor of volume V with fresh medium at a rate F. Limiting substrate is supplied in the feed at a concentration S_0. That specific growth rate μ adjusts to the dilution rate r as mediated by the extracellular unused medium, concentration S, is inherent to the system. Limits on the dilution rate between which steady state can be maintained are the minimal and maximal growth rates of the organism involved. Then $\mu = r = F/V = \ln 2/t_g$, where t_g is the generation time. Steady-state cell populations are given by $X = \gamma(S_0 - S)$, where the yield constant γ is cell mass produced/substrate utilized. Limits on the populations over which this straight-line relationship hold are set by effects of secondary limiting nutrients, metabolic products produced by the organisms, and antimetabolite content of the system. When the reactor cell population is not constant, then growth rate is related to dilution rate by the rate of population change, and $X_2 = X_1 e^{(\mu - r)(t_2 - t_1)}$ (10).

In the case of phosphate, S was both too small for direct chemical analysis and exceeded by unavoidable background levels in the feed. In such cases, the background limiting substrate S_b will produce a cell population X_b without added substrate. When in the region of linearity, substrate additions to the feed, S_a, result in increased cell populations at steady state according to the relationship $X = \gamma(S_a + S_b - S)$. The value of S can be determined from filtrate radioactivity if added substrate is partly radioactive. Neglecting small isotope effects, the ratio R between limiting substrate S and total substrate $S_0 = S_a + S_b$ will be the same as that between filtrate and total radioactivity. Then R = counts per minute of filtrate/counts per minute of feed. Solving for the extracellular limiting substrate at a specified growth rate in a system of constant background substrate

$$S = \frac{R(S_a + X_b/\gamma)}{1 - R} \quad (1)$$

Extracellular steady-state radioactivities were

compared with those of samples from the reactor medium supply line, and substrates were quantitated according to the resulting ratio.

Nutrient flux at steady state was obtained from the substrate incorporation rate, surface area per gram of organisms produced, and growth rate. If the growth rate is expressed as grams of cells produced/(gram of cells present × minute) and the yield constant as grams of cells produced/mole of substrate utilized, then the substrate incorporation rate $V = \mu/\gamma$ has the units moles of substrate/(gram of cells × minute).

Initial uptake rates. Continuous culture directly provided a medium already containing an appropriate population of organisms grown under conditions of phosphate limitation. Populations of about 2×10^8 organisms/ml were maintained at a dilution rate of 0.09 hr^{-1}. This dilution rate gave a stable population of phosphate-limited organisms growing at half their unadapted maximal rate. Portions of the resulting steady-state culture were used directly. Except in the low sodium chloride system at *p*H 4, this background phosphate was supplemented with the addition of 300 to 500 nmoles of phosphate per liter. Background phosphate alone was sufficient for the high affinity *p*H 4 system to grow. Samples from the reactor were incubated for 30 min to deplete residual extracellular phosphate (8 to 26 nM) and then tipped into tubes containing the desired initial substrate concentrations. Fresh substrate diluted the original culture volume 1 part in 10 or less. Final glassware cleaning included a 24-hr incubation with phosphate-limited continuous culture effluent, draining, and autoclaving. Phosphate uptake velocities were calculated from the slope of radiophosphate uptake curves, the initial phosphate concentration, and the dry weight of organisms used.

Radiosotopes. Isotopes were quantitated by use of scintillation spectrometry. Radiophosphate was counted as an aqueous emulsion in a Triton X-100 mixture (34) containing 1 ml of water per sample. Where phosphate was incorporated into organisms, either on filters or in suspension, a 24-hr digestion at room temperature with shaking preceded counting to increase penetration of scintillation mixture and reduce apparent self-adsorption to negligible values (*unpublished data*). Arsenate-^{73}As was counted directly in aqueous solution by use of a Picker gamma spectrometer. Orthophosphate (^{32}P-P_i) and arsenate ([^{73}As]arsenate) were obtained from Amersham Searle, Arlington Heights, Ill. They were added at sufficiently high specific activity so as to comprise less than 1% of the total substrate. About 20 nCi/ml was added to the initial uptake experiments and 5 nCi/ml was added to the continuous culture media. The *p*H of each isotope was appropriately adjusted before addition to these low buffer capacity systems.

Phosphate metabolic pools. Phosphate-limited continuous culture samples (1.8×10^8 cells in 10 ml) were allowed to accumulate radioactive phosphate by incubation with ^{32}P-P_i and carrier at 1.0 μM phosphate for 30 min. Half of the resulting loaded cells were quickly filtered off and washed with 3 ml of 1 μM phosphate medium. Accumulated phosphate was exchanged back out by incubation (5 min) in 2 ml of 100 μM phosphate medium. Filtrates from this incubation were collected to determine the amount of radioactive phosphate exchanged from inside the organisms. Other samples were heated at 60 C for 5 min prior to incubation with phosphate to provide heat-killed controls.

Phosphate pools inside cells growing at steady state were measured by similar procedures except that 10-ml samples were filtered and the washing step was omitted. Filter pads were blotted briefly after filtration and put directly in 100 μM phosphate medium. Cell-free controls prepared by this procedure gave a phosphate carryover correction of only 5% of total pool phosphate recovered, and phosphate loss during washing was avoided.

RESULTS

Results include data from some 120 phosphate-limited continuous culture runs of about 1 month each. Daily particle counts (Coulter counter) and plate counts were essentially identical. Populations consisted mostly of single cells with a few mother-daughter combinations. No reactor wall adhesion occurred; wash water from recently drained reactors was essentially cell-free. Steady states were smooth and stable, although responsive to small perturbations, either chemical or physical, such as taking a large sample from the reactor. Although small colonies with the appearance of respiratory petites appeared on spread plates from trace metal- and glucose-limited runs after a time, this problem did not occur with the phosphate-limited runs. Maximal specific growth rates did, however, show a great deal of variability. These were consistently 0.175 hr^{-1} in batch culture or during the first few days of continuous culture. However, when attempting to gather kinetic data near μ_{max}, we found that populations did not fall in a predictable way upon increasing the dilution rate. After 1 month of such gradual increase, growth rates approach the maximal growth rate of 0.4 hr^{-1} in batch culture with yeast extract medium. Culture samples from the reactor quickly reverted to the batch rate. Stopping the feed supply producing a batch reaction in the reactor gave intermediate values. Although this phenomenon is not yet understood, we mention it here because nutrient kinetic data are usually related to the maximal specific growth rate. The higher growth rate value observed is referred to as μ_{max} adapted.

Steady-state phosphate. Cell densities resulting from phosphate additions during the course of two continuous runs are shown in Fig. 1. Background cell populations resulting without added phosphate when the *p*H 4 medium

was used were relatively consistent among runs at about 2×10^4 cells/ml. As shown, additions of radioactive phosphate to the feed had no observable effect on steady-state populations. When additional phosphate was quantitatively added to the medium, cell population increase was linear with phosphate additions up to 2 μM phosphate. Table 1 summarizes resulting yields, dry weight, and cell volume data at various dilution or growth rates. These data were used to account for background phosphate in the medium. Extracellular phosphate concentration at specific dilution rates was then calculated according to the isotope ratio between reactor filtrate and total radioactivity according to equation 1. Upward adjustment of *p*H raised background phosphate slightly, possibly because of solution of sorbed phosphate (18), but any such variation was accounted for by comparing filtrate radioactivity with simultaneously collected samples from the medium supply. Phosphate radioactivity filter blanks from the medium supply amounted to less than 0.5% of the total radioactivity. Medium adjusted to *p*H 7 and ultrafiltered showed no traces of phosphate precipitates.

Populations at steady state were maintained as low as possible so that the isotope activity ratio of sample to uninoculated medium was at least 0.05. No phosphate additions were required for steady-state populations to develop from the *p*H 4 medium. An addition of 0.3 to 0.5 μM phosphate to the reactor supply medium was included in the *p*H 7 and sodium chloride systems. Table 2 shows extracellular steady-state phosphate concentrations resulting at a growth rate of 0.09 hr^{-1} in various combinations of medium *p*H and sodium chloride concentrations. Lowest extracellular phosphate concentration occurred in the low NaCl system at *p*H 4. In addition to allowing more efficient phosphate utilization, these conditions minimize many potential problems, such as metal complex formation and bacterial contamination. These conditions were used, therefore, to measure limiting phosphate concentrations as

TABLE 1. *Steady-state yield of cells from phosphate at various dilution rates*

r^a	γ^b	Dry wt (pg/cell)	Vol (fliters/cell)
0.052	4.7×10^{14}	6.0	18
0.088	4.0×10^{14}	7.0	21
0.140	4.0×10^{14}	7.0	21

[a] Continuous culture dilution rate (feed rate/reactor volume), hr^{-1}.

[b] Yield constant at specified dilution rate, γ, as number of cells produced per mole of phosphate utilized.

TABLE 2. *Effect of pH and NaCl on steady-state[a] phosphate concentration*

Medium	Added P_i (nmoles/liter)	Population (cells/ml)	Counts per min per ml		S^b (nM)
			Medium	Filtrate	
*p*H 7 + NaCl[c] ..	500	24×10^4	734	39.9	26
*p*H 7	500	21×10^4	705	39.9	23
*p*H 4 + NaCl[c] ..	300	22×10^4	465	20.6	24
*p*H 4	0	3.2×10^4	772	78.1	8[d]

[a] Dilution rate was 0.09 hr^{-1}.

[b] Average of triplicate samples from two or more determinations of extracellular steady-state phosphate.

[c] Sodium chloride concentration was 2%.

[d] Standard deviation is 3 nM.

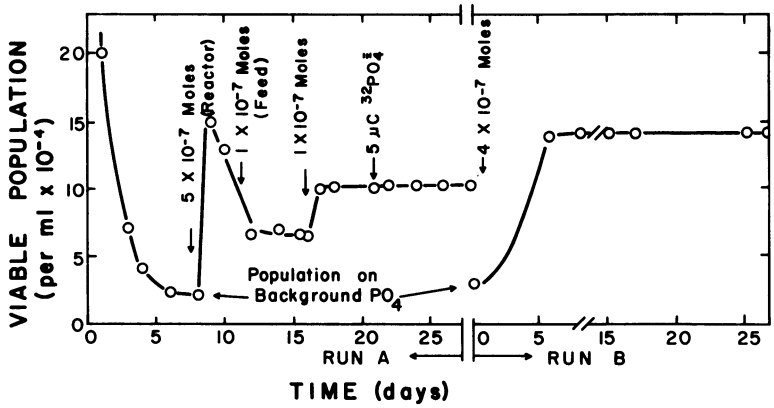

FIG. 1. *Cell populations during two continuous culture runs of R. rubra. Arrows show times of phosphate additions, giving increases in reactor or feed concentrations of molarities shown.*

a function of growth rate. Figure 2 shows average external concentrations at a series of dilution rates. Since data following show the system to be far from saturation, average concentration data of Fig. 2 are fit with the straight line $V = k \cdot S$. From a weighted least squares computer program, k is 0.026 liter nmole^{-1} hr^{-1} with an S intercept at 3.3 nM. The concentration at half maximal growth rate, $K_{0.5}$, is 6.6 to 10.8 nM, depending on the value chosen for μ_{max}. As mentioned earlier, μ_{max} gradually increases with time in continuous culture when using minimal media from 0.175 to 0.38 hr^{-1}.

Phosphate transport capacity. Phosphate accumulation capacity was sufficient for growth at very low concentrations. Figure 1 indicates extended steady state at 0.1 μg of phosphate/liter. This might be accomplished either by a transport system at high concentration in the yeast's envelope or by one of unusual affinity for phosphate. The kinetics of phosphate transport were examined to distinguish between these two possibilities. Initial phosphate uptake rates were measured to establish transport velocities at phosphate concentrations sufficiently high for saturation of the rate-limiting transport component. However, these uptake rates were in agreement with continuous culture incorporation rates only when undisturbed continuously growing organisms taken directly from the reactor at steady state were presented with labeled substrate. Both batch-grown cells and continuously grown cells subjected to perturbations, such as *p*H shifts, ionic strength changes, or harvesting, produced greatly reduced uptake velocities. Figure 3 shows typical discrepancies resulting. Clearly, growth and harvesting conditions are as important to the extent of phosphate uptake capacity of the organisms as is the suspending medium. Since changing conditions from those used for growth to those used for transport studies produces depressed rates, transport measurements based on initial uptake rates can be misleading. In subsequent experiments, continuous culture supply medium was prepared so that only substrate concentration required adjustment prior to measurements of the isotope incorporation rate. Changes in the incubation time (normally 30 min) used between sample collection and uptake measurement did not significantly alter phosphate transport rates. Figure 4 shows these initial rates of phosphate uptake as a function of concentration in the *p*H 4 system and *p*H 7-sodium chloride medium. Data are replotted as their logarithms for separation at the important low end of the substrate concentration range. Data reported on the phosphate initial uptake rate are limited to phosphate concentrations above 0.1 μM, at which background phosphate becomes insignificant. Below this concentration, background phosphate introduces significant error. Uptake velocities at lower phosphate concentrations are calculated by use of the steady-state data from continuous culture shown in Fig. 2 together with yields from Table 1, as explained in Materials and Methods. Intermediate kinetic data are pro-

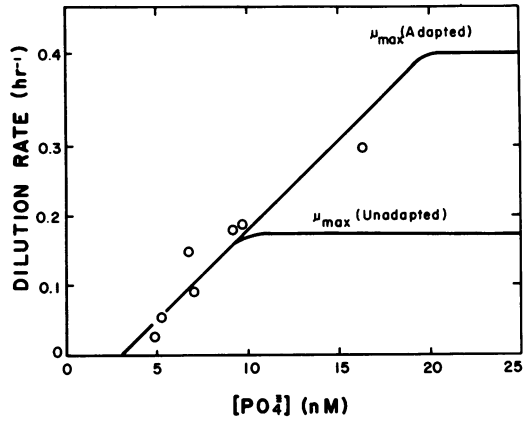

FIG. 2. *Steady-state phosphate concentration measured according to equation 1 at various dilution rates. Upper limits are μ_{max} values from batch growth curves or continuous culture wash out rates before 3 days (unadapted) or after 30 days (adapted) of operation.*

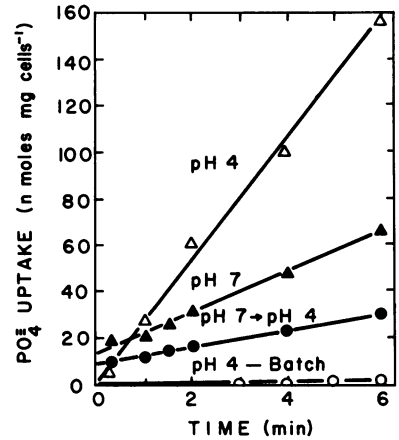

FIG. 3. *Effect of growth conditions on the initial rate of phosphate uptake. Phosphate uptake was at pH 4 with cells collected from pH 4 continuous culture* (△), *at pH 7 from pH 7 continuous culture* (▲), *at pH 4 with cells from pH 7 continuous culture* (●), *or at pH 4 with washed, phosphate-starved cells from, batch culture at pH 4* (○).

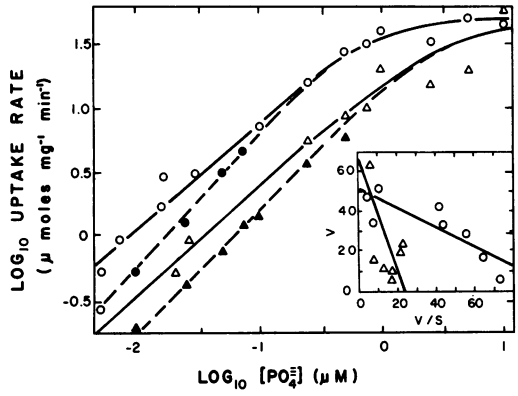

FIG. 4. *Rate of uptake of phosphate and of arsenate by cells from phosphate-limited continuous culture at pH 4 (circles) and pH 7 (triangles). Open symbols show phosphate initial uptake rates measured at or above 0.5 μM. Those below 20 nM are calculated from the steady-state phosphate data at various dilution rates shown in Fig. 2. Closed symbols show arsenate uptake rates. Inset is a replot of phosphate uptake data according to Hofstee (20).*

vided by arsenate initial uptake rates. Flux of this phosphate structural analogue could be measured at lower concentrations than for phosphate itself because background is a less significant problem (10, 35). Data at higher arsenate concentrations are limited by metabolic inhibition.

Uptake rates of both phosphate and arsenate are exceedingly high when steady-state phosphate-limited organisms are used as described. Phosphate-limited continuous culture populations of about 2×10^8 cells (14 pg, dry weight) per ml were found to accumulate about half the isotope supplied in 10 min when the phosphate concentration was below 1 μM and arsenate was below 10 nM. Higher concentrations saturated pools quickly (see below, Metabolic pools). This probably produced product and metabolic inhibition of substrate uptake. At least uptake rates began fluctuating so that shorter periods, down to 1 min, were used to filter a series of samples within the linear uptake interval. These cumulative problems of inhibition and product accumulation were probably responsible for data scatter at the higher concentrations reported necessary for significant saturation of the phosphate transport system.

The inset in Fig. 4 shows a replot of the phosphate uptake data according to Hofstee (20). This rearrangement of the Michaelis-Menten hyperbola minimizes data scatter amplification at low velocities and simplifies interpretation. Resulting maximal transport velocities were 52 and 65 μmoles of phosphate per g of cells (dry weight) per min in the *p*H 4 and *p*H 7-NaCl systems, respectively, with K_s values of 0.5 and 2.8 μM. These values agree well with the kinetic data presented below for the phosphate inhibition of arsenate uptake (K_i for phosphate is 0.7 and 1.2 μM in the *p*H 4 and *p*H 7-NaCl systems), and are probably a reasonable indication of the system's transport capacity. They compare with adapted $K_{0.5}$ and μ_{max} values of 0.01 μM and 2.0 μmoles of phosphate/(g of cells min). Sometimes initial metabolic rates are stimulated by arsenate, perhaps because arsenate uncouples energy-requiring reactions such as cytochrome-linked phosphorylations from those required for transport (3). This effect rather than separate incorporation mechanisms may be responsible for the slight difference in the slopes between the phosphate and arsenate uptake curves shown. The kinetic constants for uptake are substantially different from those for growth.

Transport inhibition. The high capacity phosphate-arsenate transport system was sensitive to metabolic inhibition at very low concentrations, as shown in Table 3. Metabolic and respiratory inhibitors such as iodoacetate and azide were indeed inhibitory at very low concentrations. Inhibition occurred at the micromolar level. The membrane-bound adenosine triphosphatase inhibitor *N,N'*-dicyclo-

TABLE 3. *Inhibition of phosphate transport in continuously grown phosphate-limited cells*

Inhibitor[a]	Concn (μM)	Phosphate uptake[b]		Relative transport rate (%)	
		*p*H 4	*p*H 7-NaCl[c]	*p*H 4	*p*H 7-NaCl[c]
None	0	26	12	100	100
Iodoacetate	10	12	6	46	50
NaN_3	10	0	1	0	8
DCCD	10	29	9	110	75
Toluene	Saturated	0	—	0	—
Arsenate	1.0	—	1	—	8
Arsenate	0.1	12	5	46	42
Arsenate[d]	0.1	15	—	58	—
Arsenate	0.01	17	13	65	110

[a] Added at 20 min during a 30-min incubation prior to isotope addition except where indicated.

[b] Phosphate uptake is expressed in micromoles per gram of cells (dry weight) per minute from a 0.5 μM initial concentration.

[c] Sodium chloride concentration was 2%.

[d] Arsenate was added at 30 min simultaneously with phosphate.

hexylcarbodiimide (DCCD; 17) was somewhat less effective. Arsenate, apparently accumulated by this very efficient phosphate transport system, was inhibitory in the nanomolar range. Effects were immediate, phosphate accumulation being reduced simultaneously with 0.1 μM added arsenate as shown. Iodoacetate and azide inhibition of arsenate transport in the high affinity *p*H 4 system was similar to that shown for phosphate transport inhibition.

Metabolic pools. Because the system exhibited both quite large transport capacity and sensitivity to membrane disruptants, one might expect appreciable metabolic pool accumulation. Accumulated arsenate or phosphate could easily be extracted by 60 C ethyl alcohol, hot water, or 10% trichloroacetic acid. Internal concentration was calculated at 0.3 mM arsenate from a 6-min exposure of continuously grown phosphate-limited organisms to 10 nM arsenate in one experiment and at 1.46 M phosphate from a 1-hr exposure to 5 μM phosphate in another. It was also noted that accumulated intracellular phosphate began to decrease after 1 to 2 min of exposure to phosphate at a concentration above 10 μM. This fall in internal phosphate was assumed to be due primarily to unbound phosphate efflux from internal metabolic pools through normal phosphate transport mechanisms because of the rapid and extensive response. Concentrations of internal free phosphate exchangeable with that outside the cell were estimated by accelerating the exchange of accumulated radiophosphate with large quantities of cold phosphate. Accumulation conditions were either those of phosphate-limited growth or short-time incubation to minimize complications arising from efflux of metabolized phosphate species. A chase of high concentration cold phosphate was used to exchange quickly with pool phosphate previously accumulated from known external concentrations. Table 4 shows the amount of phosphate liberated by organisms from phosphate-limited continuous culture samples exposed to labeled phosphate solution, initially at 1 μM, for various periods. Procedures in which heat-killed cells were used resulted in little label carryover. Exchange or chase concentrations of greater than 100 μM were found to be unnecessary. Using exchange medium containing only background phosphate (about 0.3 μM) reduced recovered phosphate by a factor of 3 as shown. A 1-min exposure to the 1 μM phosphate medium was sufficient to generate an internal concentration of 149 mM calculated from cell-free space estimations. The concentration gradient across the cell envelope at 60 min was 4 million. The phosphate concentration gradient in the *p*H 7-NaCl system, not shown, was only slightly less at 1.1×10^6.

TABLE 4. *Phosphate pool concentration in loaded cells*[a]

Uptake interval (min)	PO_4 incorporated[b]	PO_4 liberated[b, c]	External concn (nM)[d]	Internal concn (mM)
1	710	294	960	149
10	3,600	340	710	173
60	11,076	530	75	281
60 (no chase)	19,265	190	6	39
60 (heat-killed)	159	42	990	—

[a] Reaction mixture contained 1.2×10^4 to 1.9×10^4 counts/min of ^{32}P-PO_4, 1.0 μM carrier, and 1.6×10^5 to 1.9×10^5 organisms/ml from a phosphate-limited *p*H 4 continuous culture.

[b] Expressed as counts per minute per milliliter.

[c] Liberated by cells in 2.5 ml of a continuous culture sample.

[d] Calculated from initial phosphate concentration and decrease in radioactivity of filtrate.

To determine whether the formation of these large metabolic pools is normal to the growth process in dilute solution, similar isotope chase techniques were used to measure phosphate pool concentrations in organisms grown at steady state. Table 5 shows that internal phosphate concentrations of 3 to 15 mM were generated during continuous culture in various media under conditions of phosphate limitation. Steady-state external phosphate was 8 to 26 nM, again giving concentration gradients exceeding 1 million.

Inhibition kinetics. Although the low phosphate systems were sensitive to arsenate concentrations in the nanomolar range, batch cultures containing 10 mM phosphate grew in the presence of as much as 10 mM arsenate. Arsenate transport velocities were studied as a function of phosphate concentration. High capacity and affinity transport systems such as this would be of obvious advantage to organisms indigenous to dilute aquatic environments. In these dilute systems, saturation of transport components would not be significant and

$$V = k \cdot S \qquad (2)$$

where V is the transport velocity at substrate

TABLE 5. *Steady-state phosphate metabolic pool concentrations*

Medium	Cells/ml	Total counts per min per ml	Filtrate (counts/min)[a]	Feed phosphate[b] (μM)	Pool phosphate[c] (mM)	Concn gradient[d]
*p*H 7	4.3×10^8	142	8.2	0.45	3.1	1.3×10^6
*p*H 7, 2% NaCl	4.9×10^8	187	9.2	1.07	4.7	2.0×10^6
*p*H 4	2.5×10^8	162	38.3	0.51	15	1.9×10^6

[a] Radioactivity of extract from a 2.5-ml portion of a 5-ml sample containing populations specified.

[b] Calculated from cell population dry weight at 0.95% phosphorus and the relationship $X = \gamma(S_0 - S)$ at a dilution rate of 0.09 hr^{-1}.

[c] Calculated from filtrate specific activity assuming cell volume-dry weight volume (density 1.05) difference gives free space; data of Table 1.

[d] Phosphate concentration gradient across the cell envelope from steady-state data of Table 2 and pool concentrations above.

concentration S, and k is a pseudo first-order rate constant at constant cell population dependent on successful collision frequency between available transport system-limiting components T and substrate molecules. Reduced uptake velocity, V_i, due to an inhibitor, $[I]$, such as phosphate, which is also available to occupy briefly the transport system forming TI and to block temporarily the formation of the TS complex, can be expressed as $V/V_i = [T]/[T] - [TI]$. Then, in terms of the ratio of inhibitor free to unavailable transport components. $[T]/[TI] = V/V\,(1 - V_i/V)$. Assuming available T is related to inhibitor concentration $[TI]$ $[I]/[TI]$, where $[TS]$ is small as compared to $[T]$ and $k/k_i = V/V_i$, then

$$\frac{[I]}{K_i} = \frac{k}{k_i} - 1 \qquad (3)$$

This formulation facilitates analysis of transport inhibition data in the region below significant substrate (arsenate) saturation, where the rate of combination of substrate with transport system components liberated from the TI complex (system-phosphate) becomes dominant. Figures 5 and 6 show arsenate uptake rates with respect to concentration according to equation 2, as they are inhibited by a series of phosphate levels. When these data are replotted according to equation 3 (Fig. 7), they yield dissociation constants for phosphate from transport system components of 0.7 and 1.2 μM for the *p*H 4 and *p*H 7-NaCl systems, respectively, and specify the dependence of arsenate transport on phosphate concentration. Multiple arsenate transport pathways similar to those found for sugar and amino acid transport have been reported (4, 13), and these may be responsible for the slight departure from linearity at high phosphate levels where arsenate uptake is somewhat above expected values. A secondary low capacity entrance mechanism less responsive to phosphate influence as well as nonspecific absorption would give this result.

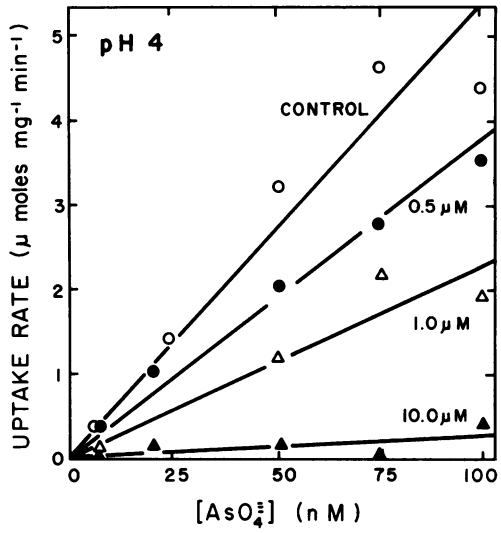

FIG. 5. *Arsenate uptake by cells from phosphate-limited continuous culture. Initial uptake medium contained phosphate as the inhibitor at concentrations shown. Arsenate concentration was varied between 5 and 100 nM. Uptake rates are micromoles of arsenate per milligram of cells (dry weight) per minute.*

In view of the high affinity of the organisms for arsenate, their sensitivity toward it, and the low K_i of competitive phosphate inhibition for arsenate transport, one would expect a major reduction in the 0.1 M arsenate concentration required to stop growth in batch culture if phosphate concentrations were also reduced to

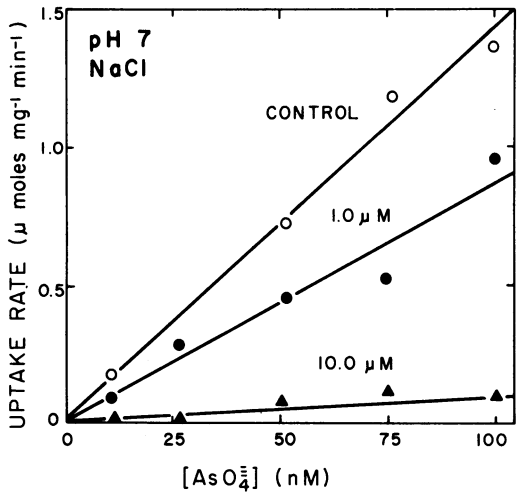

FIG. 6. *Same as Fig. 5 except that pH 7-2% NaCl medium replaced the pH 4-low salt system for both continuous culture and initial uptake.*

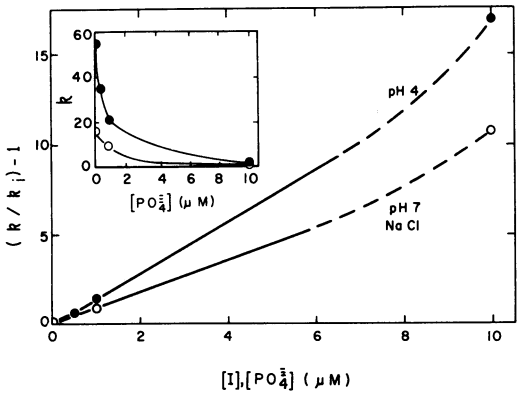

FIG. 7. *Slopes of arsenate uptake curves from Fig. 5 and 6 in the pH 4 (●) and pH 7-NaCl (○) systems according to equation 2 are shown in the inset. Data are replotted according to equation 3, giving the phosphate inhibition constant for arsenate uptake.*

very low values. This was found to be the case. Low phosphate medium was produced by incubating phosphate-limited continuous culture samples for 15 min to remove extracellular phosphate and filtering off the cells in precleaned apparatus. Filtrate with added arsenate was inoculated with continuous culture effluent, and samples were tipped out periodically for colony counts. Resulting growth curves in Fig. 8 show zero growth rates at 40 nM arsenate, with inhibition beginning in the 1 to 10 nM range. This is near the $K_{0.5}$ for phosphate of 10.8 nM, as would be expected from the similarity of kinetic constants for arsenate and phosphate uptake. The *p*H 7-NaCl system is only slightly less sensitive toward arsenate. Reduced sensitivity is probably due partly to higher residual phosphate levels inherent in the system and partly to the higher K_s and $K_{0.5}$ values, that is, lower affinities, involved.

organism, *R. rubra*, came from marine conditions of low temperature and *p*H 8, continuous cultures were easier to bring to low population steady state in low salinity systems at *p*H 4 and 25 C. Initial uptake of phosphate was reduced 90% by the addition of 350 mM NaCl prior to rate measurement. Resulting osmotic pressure changes were probably not dominant in causing this transport rate reduction because sucrose additions at equivalent molarity had no effect. Salt addition also increased phosphate concentration at steady state by factors of 0.1 and 3 at *p*H 7 and 4, respectively, as shown in Table 5. Thus, NaCl appears to be involved directly in some capacity such as effecting monovalent cation balance (14, 16, 37) during phosphate transport. High *p*H values were also inhibitory. Continuous culture samples from *p*H 4 systems showed a linear reduction in both arsenate and phosphate transport rate with increasing *p*H between 4 and 8. This amounts to 85% at *p*H 8. Some of this loss was transient, as shown (Fig. 3). Nevertheless, steady-state phosphate levels with or without NaCl were significantly higher at *p*H 7 than at *p*H 4, as

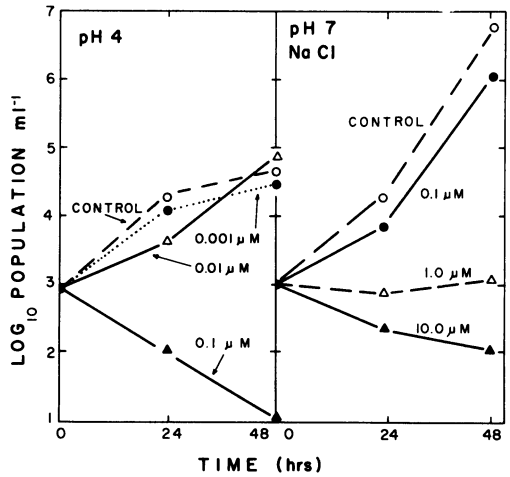

FIG. 8. *Growth curves resulting in phosphate-free medium inoculated from phosphate-limited continuous culture. Arsenate was included at time zero at molarities shown. Populations were determined from spread plates. Both continuous culture and batch growth was in pH 4 (left) or pH 7-NaCl (right) medium.*

shown in Table 2. Cumulative high salt and pH effects are shown by faster transport and lower steady-state phosphate concentrations in the low NaCl system at pH 4 than with 2% NaCl at pH 7. Initial uptake rates of phosphate were found to be 2.8 times higher at pH 4 than those in the pH 7-NaCl system (Fig. 4). The values for $K_{0.5}$ based on unadapted growth rates increased from 8 nM in the pH 4 system to 26 nM in the pH 7-NaCl system, as shown in Table 2. Thus, higher growth rates as well as higher transport rates occur at pH 4 in the NaCl systems when phosphate is limited. Maximal growth rates under conditions of nutrient saturation in a rich yeast extract medium (not shown) were found to be unaffected by these variables.

In view of the large reduction in transport rates caused by changing conditions between growth and uptake assessment, temperature effect studies should be made with organisms growing continuously at each test temperature. This is not possible above 35 C, nor did we do this at temperatures below 25 C. Phosphate transport rates at 50 C were 37% of those at 32 C with organisms grown at 25 C, indicating continued transport at well above maximal growth temperatures. Phosphate transport velocities at lower temperatures changed with a Q_{10} of 5.4 between 5 and 20 C when cells grown at 25 C were used.

DISCUSSION

Because growth rates in batch culture are normally the same as those in continuous culture with excess substrate, the gradual but very large increase in μ_{max} was unexpected. Such adaptation may be responsible for a similar but smaller discrepancy noted by Herbert, Elsworth, and Telling (19) in their classic continuous culture work. We have been surprised at the high rates indigenous marine heterotrophic populations develop in incubators at sea, and we well may be greatly underestimating maximal growth rates where conditions allow continuous propagation for extended periods.

R. rubra grows well in continuous culture with dilute triple-distilled water medium without added trace metals or phosphate. Steady-state phosphate levels, although low, were near the rate-limiting concentrations of other heterotrophic systems with extensive substrate collection mechanisms, as given by the approximation $K_{0.5} = 1/(7 \times 10^7 \gamma)$ (11). Data show that phosphate supply is adequate at above 0.01 μM. Most marine (2) and unpolluted fresh water systems (21) are between 0.1 and 2 μM in phosphate, depending on the extent of depletion concomitant with photosynthetic activity as it varies with depth. Thus, when phosphate is of dominant kinetic importance for a species such as this, it is below the usual 0.03 μM analytical detection limits (36). Such low phosphate concentrations do not commonly exist. The apparent high nutrient affinity observed appears to be achieved by producing rather large amounts of necessary transport components. That these transport components are in excess can be seen by comparing the phosphate flux at K_s where half the components are simultaneously operative with the phosphate flux required for sustained growth (from Fig. 2 and γ). At the limiting value where growth rate is maximal, and in the absence of efflux, only 4% of the transport components need function at any one time to provide phosphate at the required rate. When phosphate concentration is sufficiently low for growth rate control ($K_{0.5}$ = 6.6 to 10.8 nM), transport systems are far from saturation and velocities are essentially first order.

The original work of Monod (32) with glucose-limited continuous cultures showed small positive concentrations when growth velocities were extrapolated to zero, as noted by Mallette (28). This also appeared to be the case for phosphate. Although extensive data collection at low velocities suggested a positive intercept, the dispersion of $\pm$2.7 nM puts the origin within the standard deviation of the intercept.

Similar threshold concentrations appear in our kinetic data on glucose-limited growth (*unpublished data*). In view of the high internal phosphate concentrations measured, consistent with the millimolar values for sugars found by Kotyk and Höfer (25), and the resulting large concentration gradients across the cell envelope generated, of the order of 10^6, it is useful to consider the route by which external nutrient concentration controls growth. Nutrient-controlled growth rate is probably set by concentrations at the various sites of enzymatic activity. These are local internal concentrations and, although difficult to measure, are probably reflected by nutrient pool concentrations. If the function describing each process leading to material flow to or from these pools is known, it is an easy matter to describe growth rate as a function of internal nutrient concentration, the latter being set by a number of processes, among which is external nutrient concentration. Some of the functions describing nutrient flow between intracellular loca-

tions, including its immediate environment, are as yet undescribed. We have obtained steady-state computer solutions for growth, as controlled by external substrate concentrations and mediated through the internal nutrient pools, by equating the sum of the various nutrient sources and sinks (*unpublished data*). The model assumes that nutrients are supplied to the pools through active transport and endogenous nutrient supply, and that pool depletion is due to efflux, endogenous metabolism, and growth. The Michaelis-Menten hyperbola replaced unknown functions. Sigmoidal and hyperbolic curves, as well as the step-type curves shown in Fig. 2, result, depending on the values chosen for the various kinetic constants. Threshold concentrations always appear. These thresholds are increased by the rate of endogenous metabolism, by decreased resistance to efflux, and by increased Michaelis constants for growth. Thus, intercepts of the type shown in Fig. 2 are entirely possible. Proper equations describing overall nutrient-limited growth must contain a number of terms, at least one for each process relevant to the steady-state nutrient concentration at the sites of growth-related enzymatic activity.

The rapid establishment of the large phosphate concentration gradients found clearly requires high and directional transport rates. However, early initial uptake measurements on batch-grown phosphate-starved cells, although linear, were far too low to account for the observed growth kinetics as noted.

Cells from phosphate-limited continuous culture samples had much higher phosphate uptake rates than those from batch culture. Even centrifugal harvesting of continuously grown cells depressed rates, as noted for algae by Droop (15). That uptake rates were undisturbed by using continuous culture samples can be seen by comparing initial uptake rates with the steady-state nutrient flux in continuous culture. This agreement is shown in Fig. 4. The two methods yield rates according to equation 2 which are described by the same rate constant k. Rate constant agreement is implied by the straight line fit to the data. Initial uptake experimental ranges were extended by the use of the structural analogue arsenate and produced data in good agreement with both methods of phosphate flux measurement. The phosphate uptake rate amounts to 5×10^3 moles/(g of cells min) from medium 1 μM in phosphate. This exceeds rates estimated from the linear portions of phosphate uptake curves normalized to equivalent phosphate concentration when using published data for *Escherichia coli* (30) by a factor of 1.3 $\times 10^4$, for *Saccharomyces cerevisiae* (8) by 4.2×10^4, for *Euglena gracilis* (6) by 1.7×10^4, for baker's yeast cake (32) by 5.2×10^6, and from our own batch experiments by 5×10^2.

Although the rates measured greatly exceed published phosphate uptake data, they do not appear unreasonable. Shehata and Marr (35), using a continuous transfer process, found equivalent growth rates at a glucose concentration 100 times the phosphate concentration reported here which is just accounted for by cell yield differences between the two substrates. Moreover, usual collision frequency equations (1) that specify the concentration-dependent rate of molecule contact with a stationary plane give a lower phosphate concentration limit of 0.37 nM for providing phosphate to the cell surface at the rate it is required by organisms growing at 0.1 hr^{-1}. Our phosphate concentration measurements give a 20-fold excess in phosphate with the cell surface collision rate, neglecting electrostatic and solvent cage enhancement effects (1), to be accounted for by collisions with components in the plane of the cell surface that do not result in transport, by gradients established in Nernst diffusion layer (7, 26), and by loss of phosphate by efflux. Of course, comparisons between species and strains are dependent, among other things, on the quantity of material devoted to transport by each. Shehata and Marr (35) found high glucose transport rates in *E. coli* but comparatively low phosphate transport rates. Medveczky and Rosenberg (30), also using *E. coli*, reported much higher phosphate transport rates; yet the latter workers only found 2×10^4 molecules of phosphate binding protein per cell (29) so that the low phosphate affinity reported by Shehata and Marr (35) is possibly due to a less effective transport system.

The high capacity transport system reported here is apparently used to maintain normal metabolic pool concentrations even in dilute aquatic environments. Exchangeable phosphate recovered from phosphate-limited organisms was similar in amount to that recovered from organisms exposed to higher phosphate concentrations over a variety of times. Because of this agreement and because of the short time interval required to induce efflux (15 to 30 sec at 0.1 mM phosphate), it is unlikely that stored phosphate makes a large contribution to phosphate recovered. Although calculatations

specify average internal concentrations, both channelling and Fickian flow quite likely effect differences at reaction sites. Since resulting concentration gradients were found to be very large, of the order of 10^6, one would expect dramatic effects from metabolic inhibitors and membrane disruptants. This was found to be the case, with inhibitors generally effective in the 0.01 to 10 μM range. Sensitivity was apparent with respiratory inhibitors such as azide at lower concentration than with the membrane-bound adenosine triphosphatase inhibitor DCCD. This inhibitor is effective in *Streptococcus* at very low concentrations (17). Thus, direct linkage of transport to cytochromes (3) seems more probable for this strictly aerobic yeast than with an adenosine triphosphate-driven system. Sensitivity found toward membrane disruptants such as toluene would be expected because of the large concentration gradients involved.

This high order of sensitivity in dilute systems has interesting environmental implications. Arsenate in the marine system occurs at 0.11 μM and exceeds phosphate concentrations on occasion (22). Rain and snow have contained arsenate at 0.5 μM (24), with ground water contents about the same (38).

Arsenate competes directly with phosphate for transport. This is shown by similar rate constants for phosphate and arsenate uptake (Fig. 4) and by the ability of phosphate to reduce arsenate uptake according to equation 3 derived to fit competitive inhibition in the environmentally important region far below phosphate transport system saturation. Also, values of K_s for phosphate transport of 0.5 and 2.8 μM in the low salt-*p*H 4 and *p*H 7-sodium chloride systems, respectively, agree well with the dissociation constants of 0.70 and 1.2 μM determined from phosphate inhibition of arsenate transport.

Mutual competitive inhibition of arsenate and phosphate has been widely observed, notably by Jung and Rothstein (23); however, concentration levels reported here are significantly lower. The identical transport rate constants found predict that arsenate-phosphate concentration ratios in metabolic pools will be similar to external concentration ratios, so that arsenate toxicity is directly related to phosphate concentration. The practical significance of the K_i values for phosphate determined is that arsenate flux is halved by phosphate when arsenate is less than K_s for phosphate and phosphate is in the region of K_i, or about 1 μM. Since arsenate inhibition of growth began at 1 to 10 nM arsenate, with a zero growth rate at about 40 nM in low phosphate systems, it is probably ecologically significant that deep ocean arsenate concentrations range well above these values (22).

Salt sensitivity was apparent, although the organism used could adapt to tolerate a wide range of concentrations. Phosphate uptake kinetics and *p*H responses were such that growth could be expected to be competitive with other organisms in either marine or fresh water systems. The reason for the low *p*H phosphate transport optimum is not apparent, although it appears to be relatively common in yeast (25). Early flux data gave a good fit to the $H_2PO_4^-$ titration curve; however, transport of this single phosphate species to the exclusion of others, as proposed by Mitchell (31), seems unlikely. The second pK_a of phosphate is much higher than that of arsenate; yet our flux versus *p*H values at constant concentration curves for the two substrates spanning this range obtained by using *p*H 4-grown continuous culture samples (not shown) were nearly identical.

The advantages of continuous culture in preparing organisms of a suitable physiological state for studying kinetic effects of dilute metabolites are noteworthy. Isotope dilution-bioassay techniques developed should be equally useful for kinetic studies of other nutrients which are normally also present as low level contaminants in culture media. A subsequent communication will consider some aspects of multiple simultaneous carbon source utilization kinetics in this system.

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