CONTINUOUS CULTURE OF A MARINE HETEROTROPHIC

MICROORGANISM IN MULTIPLE SUBSTRATE SOLUTION

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CONTINUOUS CULTURE OF A MARINE HETEROTROPHIC MICROORGANISM IN MULTIPLE SUBSTRATE SOLUTION

A

DISSERTATION

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By

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ABSTRACT

Heterotrophic metabolism of multiple organic compounds, total concentration 1 mg C/l, was studied in continuous culture using a marine bacterial isolate. Limiting substrates--glucose, arginine, and glutamic acid--were quantified by enzymatic microanalyses developed with sensitivity to about 50 µg/l. The marine isolate utilized multiple substrates at low concentration simultaneously, developing increased glucose affinity over single substrate media; the steady state glucose concentration was below detection limits (<50 µg/l) probably comparable with ambient natural water levels. Apparent threshold concentrations of limiting substrates were observed under various limiting substrate conditions, and their values varied between single substrate limited and multi[illegible]substrate limited growth. Under all experimental conditions, glutamic acid was the dominant intracellular pool constituent; 9 to 16% of the dry cell material was soluble, 3 to 7% was glutamic acid. A hyperbolic response (with concentration intercept) of growth rate with intracellular soluble material as well as intracellular glutamic acid was observed in arginine limited and arginine-glucose limited growth; they followed identical patterns. Relationships were complicated during glucose limited growth.

According to the relationships among intercellular substrate concentration, flux of intercellular substrate into the organism, growth rate and the concept of a critical amount of intracellular soluble material required to sustain cell growth, the establishment of intercellular threshold concentrations is explained.

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INTRODUCTION

Marine heterotrophic microorganisms play the dominant role in the utilization (remineralization) of dissolved organic compounds. In the ocean the total concentration of dissolved organic carbon ranges from 0.3 to 2 mg C/l (Williams, 1971) and includes an unknown number of organic species present at extremely low concentrations (µg C/l or less). Heterotrophic microorganisms must possess the ability not only to concentrate the organic compounds rapidly at high concentration but also to accumulate them effectively from extremely dilute solution. Although much effort has been focused on the ability of organisms to accumulate and utilize organic compounds added to sea water (Williams and Gray, 1970; Andrews and Williams, 1971; Vaccaro, 1969), the growth kinetics of microorganisms in dilute and multiple substrate solutions have received scant attention, probably because the techniques for estimating dissolved organic compounds at µg/l levels in sea water are either unsatisfactory or altogether nonexistent and because growth kinetics in situations with more than one limiting substrate are poorly understood. The "diauxic" growth (Monod, 1949; Stumm-Zollinger and Harris, 1971; Standing et al., 1973) frequently observed in high substrate batch culture, is unlikely to occur in the ocean because the collision frequency between the substrate molecules and the organisms is extremely low and the sequential flux of substrates into the organisms is probably inadequate for their maintenance.

The purpose of this study is to gain an understanding of the mechanisms and kinetics involved in the utilization by marine microorganisms of dissolved organic compounds present as a mixture of species in very low concentrations. The concept of steady state continuous culture is employed in this study because it provides for control of the growth rate of an organism and simulates the open flow system of the ocean. To avoid dealing with the unknown number of dissolved organic compounds and inhibitors in sea water, the experiments employed a marine isolate in a chemically defined medium containing 1 mg C/l comprised of one, two, or several organic compounds. Enzymatic microanalyses of dissolved organic compounds (glucose, arginine, and glutamic acid) were developed for low concentration assay. Potential error arising from organic contaminants in the mineral medium, growth on the reactor wall, cell clumping, and sampling induced errors were subjected to detailed investigations. The problems were either avoided or reduced to levels which had no significant effect on the study. Droop's concept (1968, 1973) of cell growth depending on the intracellular substrate concentrations was applied and extended to the multiple substrate limited growth.

Organic matter in the ocean

The organic carbon of the ocean is comprised of dissolved and particulate forms, the latter contained in both organisms and inanimate particles. Dissolved organic carbon, sometimes considered to be that

which passes through a 0.45 μm Millipore HA filter or paper equivalent, is present in quantities many orders of magnitude greater than those of particulate organic carbon. The average concentrations of dissolved organic and particulate organic carbon in the ocean range from 0.2-0.8 mg C/l and 0.005-0.03 mg C/l respectively in deep water, to 0.3-2.0 mg C/l and 0.03-0.3 mg C/l in the surface water (Williams, 1971). Since the ratio of organic to inorganic materials in sea water is of the order of 10^{-4}, simple concentration procedures are impractical in the assessment of dissolved organic carbon composition. Jeffrey and Hood (1958) have evaluated several methods for the isolation of organic compounds from sea water. However, these methods are not sufficient to identify and quantitate those organic compounds present in trace amounts. In fact, only a small number of organic molecules, accounting for less than 10% of the total organic matter in the ocean, have so far been identified (Degens, 1970).

The main sources of dissolved organic carbon are (1) excretion from phytoplankton, (2) decomposition of phytoplankton and animals, (3) excretion by animals and (4) allochthonous drainage. Sieburth and Jensen (1970) reported that up to 40% of the carbon fixed was exuded by a brown alga (Phaeophyta) during desiccation and rain emersion. The largest components of excretion appeared to be low molecular weight carbohydrates, free amino acids and peptides. Ignatiades (1973) found that the amount of organic matter released by Skeletonema costatum (Greville) cleve under field conditions was seasonally variable. On

the surface, 46% of the fixed [^{14}C]organic carbon was released back to the water in winter, while in summer, only 3.5% was detected. The factors effecting the release were further studied in the laboratory under unfavorable conditions, such as low light intensity, cell senescence, and darkness (Ignatiades and Fogg, 1973). The amount of organic carbon released was considerably higher than under normal conditions. Also, the relative amount of organic matter excreted increased up to 65% with increasing phosphate, nitrate and silicate deficiency. The reasons that phytoplankton turn on the leakage processes under conditions of stress are unknown and require further investigation.

The decomposition of dead algae by heterotrophic microorganisms can be divided into three stages: (a) a very quick removal of soluble organic components, (b) a relatively rapid decomposition of labile organic constituents, and (c) a slow decomposition of refractory organic constituents. Otsuki and Hanya (1972a, 1972b) observed that the initial overall microbial decomposition of algal carbon and nitrogen could be approximated by first order kinetics. About 40% of the carbon and 25% of the nitrogen was released into solution, the released organic products consisting mainly of organic acids and amino acids.

In marine zooplankton, dissolved organic compounds may conceivably be released by true excretion, by egestion, or by diffusion. Johannes and Webb (1970) reported that most of the amino acids commonly

occurring in proteins, are released by marine invertebrates. The main source of release may come from the zooplankton grazing process. If they ingest an average of 80% of the primary production, and 10% of the ingested organic material is subsequently excreted, egested or leaked into the sea water, then we would expect about 8% of the carbon fixed by photosynthesis will find its way to the organic matter pool in the ocean. Hobbie et al. (1968) estimated the flux of free amino acid carbon due to all planktonic organisms to be between 1 and 10% of the rate of photosynthetic carbon fixation.

A considerable amount of organic matter is transported by river waters to estuaries and marine coastal waters. The sources of organic material are mainly derived from the products of vegetable matter decomposition; domestic and industrial waste waters also provide a significant amount of organic matter to the river waters. Hunter (1971) reported that the average organic concentration of English domestic waste water is about 300 mg/l of which about 100 mg/l is in soluble form and 200 mg/l is in particulate form. Most of the readily oxidizable organic compounds are decomposed bacterially as they enter the sea water.

Heterotrophic metabolism is the major fate of organic matter in the sea. This process results in the regeneration of the carbon dioxide and nutrients. Particulate organic material serves as food for filter feeders as well as for microorganisms growing on this solid phase (Johannes, 1965). Undoubtedly heterotrophic microorganisms

(bacteria, yeast, molds, and actinomycetes) are the principal users of the dissolved organic compounds because they depend on performed organic substances for their growth. Phytoplankton also assimilate some of the dissolved organic compounds, vitamins and amino acids, for their growth; however, the amount is far less than that utilized by heterotrophic microorganisms (Provasoli, 1963; Hellebust, 1970).

Heterotrophic microorganisms of coastal and estuarine waters (Williams and Gray, 1970; Andrews and Williams, 1971) and even the arctic sea waters (D. K. Button unpublished data) have substantial potential for oxidation of compounds such as glucose, amino acids and hydrocarbons. In the English Channel, no marked seasonal variations in the concentration of amino acids and glucose were observed (Williams and Gray, 1970). However, a pronounced seasonal change in the rate of oxidation of these compounds was observed, especially with glucose (0.5-6 µg/l) for which the oxidation rate was 1% per day in the winter and 20-50% per day in the summer (Andrews and Williams, 1971). All these observations indicate that in the ocean the utilization of dissolved organic compounds by the heterotrophic population is a vigorous and significant process. However this fact creates a puzzling question; if the heterotrophic microorganisms have such a high affinity for dissolved organic matter, why do considerable quantities of labile organic carbon remain in the water throughout the year? Webb and Johannes (1967) estimated that in one month zooplankton release a total amount of dissolved amino acids equal to

that amount usually found in sea water. The level of dissolved organic concentration in the sea water is probably due to the achievement of a steady state between the input of dissolved organic compounds into the system and their utilization by the heterotrophic microorganisms for growth.

Growth of microorganisms in the ocean

The growth kinetics of marine heterotrophic microorganisms in the ocean have not yet been described. It is important and interesting to understand how these organisms grow in such a dilute, multiple substrate solution. Apparently, marine microorganisms have the ability to utilize a number of the limiting substrates simultaneously rather than sequentially. The classical microbial growth equation, the Monod equation (Monod, 1949), is invalid in this system because it is applicable only to a single limiting substrate condition. Jannasch (1967) studied the growth of marine bacterial isolates in continuous culture. He observed the existence of threshold concentrations of lactate, glucose, and glycerol which were used as limiting carbon sources for the study; however, the threshold concentrations he observed were much higher than the concentrations of organic carbon reported for sea water. The reason for threshold substrate concentrations is not known; but it is possibly due to a combination of endogeneous metabolism, leakage and intracellular metabolic control.

In 1947, Gale found that there was a pool of free amino acids in Streptococcus faecalis and the amount of this pool material depended to a certain extent upon the growth medium and the age of the culture. Since then, the composition of amino acid pools in bacteria, yeasts, molds and fungi has been actively investigated (Britten and McClure, 1962; Holden, 1962).

Brown and Stanley (1972) studied the factors affecting the nature of the microbial pool of 13 strains of marine bacteria under carefully controlled conditions. They demonstrated a number of relationships relevant to the pool concentrations in marine bacteria including the effect of growth rate and the effect of NaCl levels on pool concentration. Clark et al. (1972) as well as Tempest et al. (1970) and Gale (1947) found that in the bacterial pools there is a substantial amount of glutamic acid and its level is highly sensitive to the experimental conditions.

In the development of the "cell quota" concept, Droop (1968, 1973) pointed out that growth rate is obviously influenced by intracellular nutrient concentration. He used ^{57}Co-labeled vitamin B_{12} limited continuous cultures and based kinetic measurements on the population data and radioactivity of the labeled vitamin B_{12} removed from the culture filtrate by charcoal adsorption. The concept of a positive relationship between growth rate and internal nutrient supply was extended to nitrogen limitation of phytoplankton by Caperon and Meyer (1972).

The concept that the regulatory mechanisms which control intermediary and macromolecular metabolism interact to regulate bacterial growth was further discussed by Nierlish (1974). Intracellular regulators control the formation and arrangement of the many low molecular weight compounds that are intermediates in the formation of the macromolecular components of the cells. These low molecular weight compounds must represent the balance between the amount passing through the cell membrane from the external environment and the amount being metabolized either by breakdown or by synthesis into cell material. This further implies that the concentration of pool material is a control parameter for cell growth.

A simplified growth model of heterotrophic microorganisms in multiple substrate limiting medium is considered as follows: in a well mixed culture, the substrates are homogeneously distributed. A stagnant aqueous film surrounds the cell membrane, through which the substrates have to diffuse before they can reach the active transport systems at the cell membrane. The active transport systems are either constitutive or inducible. Constitutive transport systems are present at the cell envelop at all times, whereas inducible transport systems are induced only when they are called upon to accumulate their respective substrates. The transport systems are subjected to all sorts of inhibition (competitive, non-competitive or uncompetitive) by chemical substances present in the medium. The ability of a cell to accumulate a particular substrate depends on the availability of an

appropriate transport system for that substrate. Within a cell, there is an array of enzymes, some free in solution, some organized on particulate structures, but each under the control of a specific structural gene. A substrate after entering a cell may remain unchanged and be incorporated into macromolecules; it may be metabolized to provide energy for cell growth; or it may be transformed into other metabolic intermediates essential for macromolecular synthesis. The effective local concentration of each metabolic intermediate is under well designed control. The enzymes which catalyze metabolism, thereby providing required amounts of metabolic intermediates, are generally under feedback or product regulation. The incorporation of metabolic intermediates into macromolecules takes place in a precise genetically controlled sequence. Most likely these low molecular weight metabolic intermediates are stored in organelles known as compartmental pools which are probably separated from the main sites of metabolite interconversion and macromolecular synthesis. The pool substrates are in equilibrium with the substrates in the cytoplasm. These "loose substrates" in the cytoplasm are utilized for cell growth and are replaced by the substrate from the compartmental pools. Cell growth is controlled by the concentration of the metabolic intermediates in the pools as well as the amount of "loose substrates" in the cytoplasm. Pool concentrations are provided by the intercellular substrates through enzymatic reactions. Therefore, the concentrations of the intercellular substrates, through their ability to maintain the pool

concentrations, control the growth rate of the cell. The concentration of individual limiting substrate at steady state in a continuous culture will depend on its biochemical nature and the enzymatic systems of the cell. Obviously, a cell will select the suitable substrates from the environment to fit into its intracellular pool substrates requirement.

This simplified growth model leads one to believe that growth kinetics of microorganisms in multiple substrate limiting media are very complicated. In a single substrate limiting medium, the pool sizes are set solely by the limiting intercellular substrate concentration. Therefore, the growth kinetics of microorganisms under single substrate limiting conditions are predictable from the intercellular substrate concentration. Perhaps, the classical emprical microbial growth equation, the Monod equation (Monod, 1949) is a special case which must be reserved for organisms growing under conditions of single substrate limitation.

Continuous culture, its theory and application

Monod (1949) did a thoughtful study of microbial growth with regard to the concentration of a single limiting substrate. He observed a similarity between this relationship and the well-known Michaelis-Menten equation. The Monod equation describes the growth of the cell as a function of a limiting substrate:

$$\mu = \mu_{max} \frac{A_{out}}{K_{0.5} + A_{out}} \qquad (1)$$

The growth rate (μ) is given as a function of the concentration (A_{out}) of a single limited component of the medium. The rest of the substrates are held constant or are present in excess. The two constant parameters are μ_{max}, the maximum growth rate, and $K_{0.5}$, the substrate concentration at $\mu_{max}/2$. Monod's equation has been widely applied and has often proved satisfactory, but a number of limitations have been reported (Powell, 1967). Herbert et al. (1956) developed the continuous culture technique for examining the relationship between the growth rate, μ, and the limiting substrate concentration, A_{out}, under steady state conditions. Since then continuous culture has been used in physiological studies involving microorganisms primarily because environmental conditions can be kept constant, in sharp contrast with batch culture cultivation where microorganisms are subjected to continual change.

A continuous culture system is an open flow system which is nothing but a continuous flow stirred tank reactor. It has a culture vessel to which fresh medium is supplied and from which culture is removed at a rate of F liters per hour. Consider a culture vessel holding V liters of medium, the dilution rate of the culture medium is designated as r which is defined as the flow rate F, divided by the culture volume V. If a culture in the reactor grows at a rate faster than the dilution rate, a net increase of cell mass will be observed.

If the dilution rate is faster than the growth rate, there will be a wash out of cells in the reactor. In both cases, a steady state will be reached (if $r \leq \mu_{max}$); the culture will grow as fast as the dilution rate, and the cells in the reactor will remain constant. The above phenomena may be formulated mathematically as follows:

Cell balance

Rate of change of cells in the reactor	=	Rate of input of cells into the reactor	-	Rate of output of cells from the reactor	+	Rate of growth of cells in the reactor

$$V \frac{dX}{dt} = 0 - FX + \mu XV \qquad (2)$$

where:

V = reactor volume, liter.

X = cell mass, g/l.

t = time, hr.

F = flow rate, liters/hr.

μ = specific growth rate, hr^{-1}.

Since no cells are in the fresh medium, the rate of input of cells to the reactor is zero. At equilibrium or steady state the cell concentration in the reactor does not change, and Equation (2) becomes:

$$\mu = F/V \qquad (3)$$

Since $r = F/V$, therefore

$$\mu = r \tag{4}$$

Equation (4) indicates that the growth rate is equal to the dilution rate at steady state and it can be set at any desired value between 0 and μ_{max} by suitable adjustment of the flow rate F. Equation (4) does not tell us the relationship between μ and the limiting substrate concentration, A_{out}.

Single limiting substrate mass balance:

A similar mass balance equation can be performed on the sole limiting substrate:

Rate of change of substrate in the reactor = Rate of input of substrate into the reactor − Rate of output of substrate from the reactor + Rate of substrate utilized for growth in the reactor

$$V\left(\frac{dA_{out}}{dt}\right) = FA_o - FA_{out} - V\left(\frac{dA_{out}}{dt}\right)_{\substack{\text{consumption}\\ \text{for growth}}} \tag{5}$$

$$\left(\frac{dA_{out}}{dt}\right)_{\substack{\text{consumption}\\ \text{for growth}}} = \frac{1}{Y_{aA}}\left(\frac{dX}{dt}\right) = \frac{X}{Y_{aA}}\left(\frac{1}{X}\frac{dX}{dt}\right) = \frac{X\mu}{Y_{aA}} \tag{6}$$

where Y_{aA} is defined as the yield constant, gram of cell produced per gram of limiting substrate utilized, that is, $X/(A_o - A_{out})$, at a given

growth rate and A_o is the concentration of the limiting substrate in the fresh medium. Substituting Equation (6) into Equation (5) at steady state when $\frac{dA_{out}}{dt} = 0$, Equation (5) becomes:

$$F\left(A_o - \tilde{A}_{out}\right) = \frac{\mu \tilde{X} V}{Y_{aA}} \tag{7}$$

$$r\left(A_o - \tilde{A}_{out}\right) = \frac{\mu \tilde{X}}{Y_{aA}} \tag{8}$$

where:

$\tilde{X}$ = steady state cell mass, g /l.

$\tilde{A}_{out}$ = steady state limiting substrate concentration, g /l.

Since at steady state from Equation (4) $\mu = r$, Equation (8) can be written:

$$\tilde{A}_{out} = A_o - \frac{\tilde{X}}{Y_{aA}} \tag{9}$$

indicating that one can estimate the concentration of limiting substrate at steady state by just measuring the cell mass if the yield constant and the true value of A_o are known. Practically, $\tilde{A}_{out}$ is estimated directly from the filtrate rather than from Equation (9). By estimating the steady state value $\tilde{A}_{out}$ at μ between 0 and μ_{max}, one can obtain the relationship between the specific growth rate of

the microorganism and the limiting substrate concentration in the medium.

MATERIALS AND METHODS

Organism

A marine bacterium, the 198th isolated from Cook Inlet, Alaska, was selected for this study. It is a gram-positive, aerobic, small rod shaped (0.6x3 μm) and diphasic (i.e., it occasionally produces a scalloped colony on spread plates) bacterium which can oxidize hydrocarbon and grow well at pH 7. It liquifies gelatin but does not grow on starch. The taxonomy of this organism is under investigation; it is referred to here as isolate 198.

The reasons for choosing isolate 198 for this work were its ability to metabolize numerous substrates and its ease of maintenance in long term laboratory culture. Table 1 shows that growth occurred on 17 out of 23 carbohydrates tested[1], and the addition of amino acids to the various monomers produced substantially more dense populations. Table 2 indicates that 7 out of 20 amino acids tested as sole carbon sources supported growth. In addition, growth was substantial with either single or multiple hydrocarbons as the sole carbon source. Spread plates grew well on vapors from dodecane. Growth in liquid culture with kerosene as the sole carbon source was detected by the evolution of carbon dioxide. Figure 1 shows a chromatogram of residual components after cultivation of the isolate in kerosene. The peaks

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[1]Unpublished data provided by D. K. Button.

Table 1. Carbohydrates utilized by isolate 198.

Substrate	Growth[1]	Substrate	Growth[1]
Erythritol	-	Melezitose	+
Inulin	-	Rhamnose	+
D-arabinose	+	Salicin	++
L-arabinose	+	Inositol	+
Galactose	+	Sucrose	+
Mannitol	+	Dextrose	+
D-xylose	+	D-fucose	+
L-xylose	-	Galactonolactone	-
Glycerol	+	Glucose	+
Mannose	++	Fructose	-
Lactose	-	Galacturonate	+
Melibiose	+		

[1]Growth was estimated by measuring the optical density at 625 mu after 72 hours of incubation.

Table 2. Growth on individual amino acids.

Substrate	Growth[1]
Leucine	++
Arginine	++
Valine	++
Glutamic acid	+
Isoleucine	+
Alanine	+
Asparagine	+
All others	-

[1]Growth was estimated by measuring the optical density at 625 mu after 72 hours of incubation.

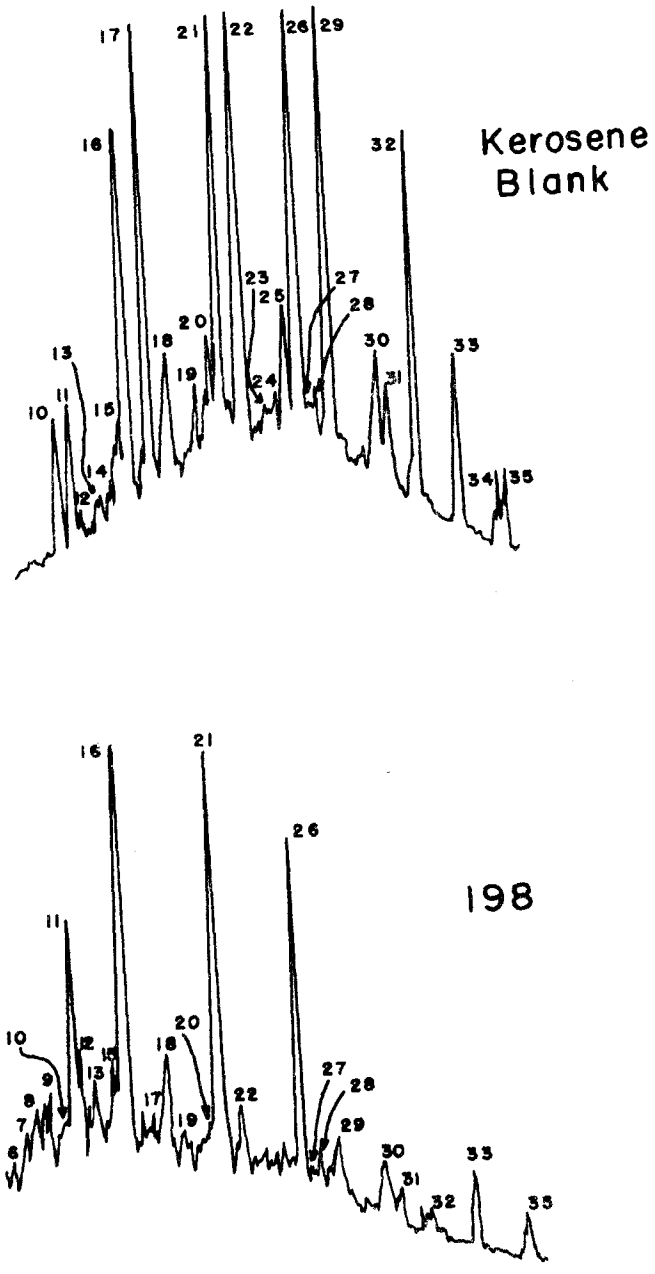

Figure 1. Kerosene utilization by isolate 198 in batch culture. Gas chromatograms of pentane extract from kerosene saturated medium with (below) and without (above) cells. Those peaks utilized are assigned approximate chain length from known standards and are listed in Table 3. (D. K. Button unpublished data).

utilized are assigned approximate chain lengths from known standards and are listed in Table 3[1].

Isolate 198 grows on rubber tubing and black rubber stoppers. Even the contaminating organics in distilled water and in reagents can support growth to a population of about 10^5 cells/ml. It has a tendency to clump at high cell populations (>10^6 cells/ml) so viable count and electronic particle (coulter) counter techniques could not be applied for estimating cell mass. Eventually, a ^{14}C-method was employed for estimating the cell mass (see analytical techniques) using ^{14}C-uniformly labeled organic substrates.

Chemicals

All chemicals were reagent grade, supplied by Baker Chemical Co., Phillipsburg, N. J., and Sigma Chemical Co., St. Louis, Mo. All organic compounds used in this study were ^{14}C-uniformly labeled. L-arginine monohydrochloride (10 mCi/mmole), L-glutamic acid (10 mCi/mmole), D-glucose (3 mCi/mmole) and amino acid mixture (57 mCi/mAtom carbon) were purchased from Amersham Searle Corp., Arlington Heights, Illinois. Urea (40-60 mCi/mmole) was obtained from Catalonic Inc., Los Angeles, California.

Enzymes

Arginase (powder, A-2137, 60 units/mg), luciferin-luciferase

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[1]Unpublished data provided by D. K. Button.

Table 3. Components utilized by isolate 198 from kerosene.

Carbon #	Peak	Utilized
11	5	
12	10	+
	11	
	16	-
13	17	+
	21	-
14	22	+
	26	-
15	29	+
	30	-
16	32	+
	33	-
17	34	+
	Pristane	+
	Dodecane	+

(FLE-50, firefly lantent extract), glucose oxidase (G6125,13-18 units/mg), hexokinase (type III, H5000, 12-16 units/mg, Lot No 34C-8140) and L-glutamic decarboxylase (G2001, 0.45-0.75 units/mg) was obtained from Sigma Chemical Co., Urease (Code URC, 50 units/mg) was supplied by Worthington Biochemical Co., Freehold, N. J.

Liquid scintillation solutions

Except for the determination of [^{14}C]carbon dioxide in an aqueous solution of [^{14}C]carbonate which was counted in a liquid scintillation solution composed of 2 g 2,5 diphenyloxazole (PPO), 25 mg 1,4 bis-2-(5-phenyl oxazolyl-benzene) (POPOP), 500 ml toluene and 500 ml methanol (Yardley, 1964), all ^{14}C-radioactive samples were counted in a fluid containing 900 ml Triton X-100 (Baker), 2,100 ml toluene, 12 g PPO, and 0.3 g POPOP.

Medium

The chemically defined medium, adjusted to pH 7.0, had the following composition per liter of double glass distilled water: NaCl, 10 g; $NaH_2PO_4 \cdot H_2O$, 1 g; K_2HPO_4, 1 g; $(NH_4)_2SO_4$, 60 mg; KCl, 40 mg; $MgSO_4$, 25 mg; $CaCl_2 \cdot 2H_2O$, 5 mg; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 0.7 mg; $CuSO_4 \cdot 5H_2O$, 25 mg; $MnSO_4 \cdot 7H_2O$, 1.5 mg; $CoSO_4 \cdot 7H_2O$, 1.5 mg; MnO_3, 0.5 mg; vitamin B_1, 5×10^{-10} moles; vitamin B_{12}, 5×10^{-10} moles; biotin, 5×10^{-10} moles. In addition a limiting organic substrate was added. All the above components except $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ which

precipitates when heated with the other substances, and the limiting organic substrate were combined. It was necessary to remove as much organic contamination as possible. Organic contaminants in such a medium are able to support the growth of isolate 198 in batch culture and in continuous culture to a population of 10^5 cells/ml. Treatment of the medium with ultraviolet light (Button, 1968) only removed some of the contaminants, so a technique for removing most of the growth supporting compounds from the medium was developed. Five ml of an "organic carbon free" culture of isolate 198 containing about 1 mg/l of cells (dry) were added to a pyrex carboy containing 20 liters of the medium to be treated. A magnetic stirrer and a 3" magnetic bar were used to mix the batch culture for one week, then the culture was filtered at low vacuum pressure (2/3 atmospheric) through a 0.45 μm membrane filter previously rinsed with boiling water to remove any soluble organic contaminants. The whole filtration system was made up of pyrex glass capillary tubing and carboys, all parts having been cleaned with dilute hydrochloric acid and rinsed with double glass distilled tap water.

Figure 2 shows the continuous culture population levels reached by isolate 198 in both a medium treated as just described and in an untreated medium. It clearly indicates that most of the organic contaminants in the medium are removed by the above treatment. Those remaining have a negligible effect on the measurement of intercellular substrate concentration. If we assume that the average dry weight of

isolate 198 is 10^{-13} g/cell the remaining substrates can introduce a maximum error of only 0.5 μg/l in the cell mass determinations in this study.

The treated incomplete medium was autoclaved for one hour at 15 psig steam pressure and allowed to cool to room temperature. The desired amounts of limiting organic substrate and the $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, autoclaved separately in sealed ampoules, were added to the cooled medium in the carboy which was then agitated vigorously for about fifteen minutes to insure complete mixing as well as restore to the medium the dissolved oxygen removed during autoclaving. At this point the sterile medium was ready to be used for continuous culture. The organic compounds used in this study totaled about 2.5 mg/l (1 mg C/l) and usually contained 1-1.4 μCi/l of the ^{14}C-uniformly labeled carrier.

Continuous Culture Apparatus

A single phase continuous culture system (Button and Garver, 1966) was first employed for this study. However, isolate 198 is able to work its way upstream and infect the fresh medium source in such a system. It also thrives on black-rubber stoppers. Consequently, a two phase system was developed to avoid the organism's contact with the fresh medium and with the rubber stoppers.

The two phase continuous culture system consists of a 20 liter pyrex carboy, a modified flat bottom flask (250-2,000 ml) and a precision volume displacement pump (controlled volume miniPump, Milton Roy Co., St. Petersburg, Florida) connected in the above order with glass capillary tubing and rubber tubing as shown in Figure 3. The fresh medium feed line is made up of all glass capillary tubing except for a 1" piece of rubber tubing[1] (Amber Latex, VWR Scientific) used to make the final feed line connection after autoclaving. In the reactor, the culture has no contact with the rubber stoppers and the fresh medium feed line. Clamp C (see figure 3) is open only at the beginning of the experiment while the reactor is being filled to the selected volume with fresh medium from the carboy. It is closed for the rest of the experiment.

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[1]This short section of rubber tubing is cleaned with 0.1N HCl and rinsed with hot distilled water to reduce the labile organics on the inside surface.

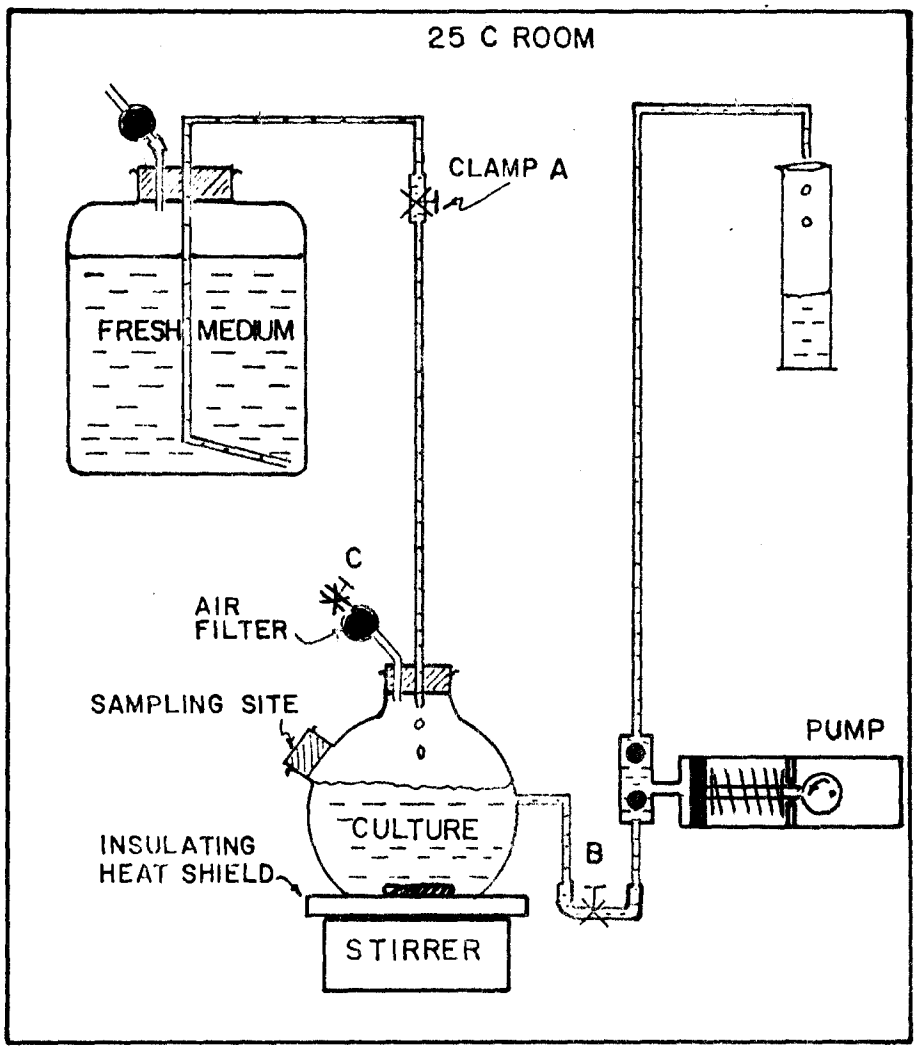

Figure 3. Continuous culture system.

This system was designed to operate at low cell populations (<5 mg/1). The yield constant of oxygen (g cell produced per g of O_2 consumed) is about one, and the solubility of oxygen in the medium is about 8 mg/1 at room temperature; therefore, it is not necessary to bubble air through the reactor during the experiment.

Continuous Culture Operation

A. Sampling procedure

Samples were withdrawn from the sampling site with a sterilized disposable syringe and needle. The sampling procedure was as follows: clamp A and then clamp B were closed (see Figure 3); the rubber stopper at the sampling site was flamed, the needle inserted, and the sample withdrawn. After sampling, clamp B and then clamp A were released. Fresh medium replaced the amount of sample removed. To avoid disturbing the culture conditions in the reactor, not more than 1.5% of the culture volume was withdrawn each time, and only one sample was taken in a day.

B. Sample treatment

The sample (2-10 ml) was immediately filtered through a 0.45 μm millipore filter (HAWP 013 00, Millipore Corp., Bedford, Massachusetts). The filtrate was collected in a cleaned test tube to which had been added one drop of 6N HCl. The tube was placed in a hot water bath

(95-100°C) for 2-3 minutes to stop the enzymatic reactions. It was then immediately cooled to room temperature to avoid loss of water by evaporation and was stored below -15°C until analyzed. The samples were normally analyzed immediately or within five days. No significant loss of arginine or glucose was detected after two weeks of storage.

Immediately after separating the cells from the filtrate, 10 ml of 1% saline solution (pH 7) were passed through the filter to remove the ^{14}C organic substrate adsorbed onto the filter, the filter was blotted on a piece of paper towel and immediately transferred to a glass scintillation vial (20 ml, Kimble) to which 10 ml of scientillation fluid were then added. The cell mass was estimated as described in the analytical techniques section.

C. Sampling induced errors

Substantial errors in obtaining the steady state limiting substrate concentration can be caused by simple oversights in sampling methods and protocol. Three such errors are considered: substrate depletion during sampling; sample contamination with unequilibrated fresh medium; and reactor wall growth.

I. Sample substrate depletion in the time interval between sample removal from the fermentor and metabolism arrest in the sample.

The initial substrate removal rate in a continuous culture sample is the same as the rate that substrate is utilized in the reactor. If the culture has a high substrate affinity, then the substrate decay

rate in the sample can be very fast. Whether this type of decay is sufficient to induce a serious error in a given type of sampling procedure can be computed from the following derivation:

At steady state, the cell mass and the limiting substrate concentration in the reactor are designated as $\tilde{X}$ and $\tilde{A}_{out}$ respectively. Let the cell mass and the limiting substrate be X and A_{out} in the sample drawn from the reactor after time, t. Then

$$X - \tilde{X} = Y_{aA}(\tilde{A}_{out} - A_{out}) \tag{10}$$

and

$$X = \tilde{X} + Y_{aA}(\tilde{A}_{out} - A_{out}) \tag{11}$$

where Y_{aA} is the yield constant as previously defined. Differentiating Equation (11) with time, we obtain

$$\frac{dX}{dt} = - Y_{aA} \frac{dA_{out}}{dt} \tag{12}$$

Dividing Equation (12) by X,

$$\frac{1}{X}\frac{dX}{dt} = - \frac{Y_{aA}}{X}\frac{dA_{out}}{dt} \tag{13}$$

Since $(1/X)(dX/dt) = \mu$, substituting Equation (11) into Equation (13)

yields

$$\mu = -\frac{Y_{aA}}{\tilde{X} + Y_{aA}(\tilde{A}_{out} - A_{out})} \frac{dA_{out}}{dt} \qquad (14)$$

Rearranging Equation (14),

$$\frac{dA_{out}}{\tilde{X}/Y_{aA} + \tilde{A}_{out} - A_{out}} = -\mu\, dt \qquad (15)$$

Assuming the growth rate of the cell is constant and is the same as in the reactor and then integrating Equation (15), we obtain

$$\ln(\tilde{X}/Y_{aA} + \tilde{A}_{out} - A_{out}) \Big|_{\tilde{A}_{out}}^{A_{out}} \Big|_{t=0}^{t=t} = \mu t$$

$$\ln\left[\frac{\tilde{X}/Y_{aA} + \tilde{A}_{out} - A_{out}}{\tilde{X}/Y_{aA}}\right] = \mu t$$

Therefore,

$$t = \frac{1}{\mu} \ln\left[\frac{\tilde{X}/Y_{aA} + \tilde{A}_{out} - A_{out}}{\tilde{X}/Y_{aA}}\right] \qquad (16)$$

Equation (16) specifies the time, hr, required for the limiting substrate in the sample to drop from the steady state value $\tilde{A}_{out}$ to a new value A_{out}. The time required for decaying half the value of $\tilde{A}_{out}$ is as follows:

$$t_{1/2} = \frac{1}{\mu} \ln \frac{\tilde{X}/Y_{aA} + (1/2)\,\tilde{A}_{out}}{\tilde{X}/Y_{aA}} \qquad (17)$$

If $\tilde{X}/Y_{aA} >> \tilde{A}_{out}$, Equation (17) indicates that it would not take long for the cells to deplete the limiting substrate in the sample. In this work, the cell mass was less than 1 mg/l, and the limiting substrate value $\tilde{A}_{out}$ was between 0.05 and 1.0 mg/l. For example, in an arginine limited continuous culture,

$$\tilde{X} = 0.5 \text{ mg/l}$$

$$Y_{aA} = 0.25$$

$$\mu = 0.2 \text{ hr}^{-1}$$

$$\tilde{A}_{out} = 0.1 \text{ mg/l}$$

Substituting these data into Equation (17),

$$t_{1/2} = 7.4 \text{ min.}$$

This calculation indicates that if the above sample were withdrawn from the reactor, and allowed to sit for 7.4 minutes without removal

of the cells from the medium, a 100% error in estimating the steady state limiting substrate concentration would be introduced. If the time lag were reduced to 0.5 minutes, from Equation (16) and the above data, the error in estimating $\tilde{A}_{out}$ would be only 4%. The sample treatment used in our experimental procedure required about 30 seconds, so theoretically, an error of about 5% was introduced in the limiting substrate concentration estimation.

In laboratory culture studies, the cell mass is often very high. Let us consider the following information,

$$\tilde{X} = 2.0 \text{ g/l}$$

$$\mu = 0.5 \text{ hr}^{-1}$$

$$\tilde{A}_{out} = 10 \text{ mg/l}$$

$$Y_{aA} = 0.5$$

The time required for $\tilde{A}_{out}$ to drop to zero can be estimated by substituting the above information and $A_{out} = 0$ into Equation (16). From the calculation, the time required is 18 seconds. This may explain the fact that threshold concentrations for intercellular substrate are frequently not observed and that this ambient substrate concentration presents an obscure relationship with growth rate.

II. Sample contamination due to unequilibrated medium.

Erroneous estimations of continuous culture steady state limiting substrate concentrations can result if a little fresh medium, high in

limiting substrate, is included in the reactor sample.

The following derivation shows the error introduced by this sort of contamination. If during sampling (see Figure 3 and the sampling procedure) clamp A is left open, fresh medium will continue to flow into the reactor while the sample is being withdrawn. If we assume that there is instantaneous mixing as the fresh medium enters the reactor while the cell mass remains constant, from the limiting substrate mass balance in the reactor, we can write the following equation:

$$V \frac{dA_{out}}{dt} = QA_o - QA_{out} \tag{18}$$

Where Q is the sample withdrawal rate, ml/sec., t is the time interval of sample withdrawal (sec), and V is the reactor volume (ml). By integrating,

$$\ln (A_o - A_{out}) = -Wt + c \tag{19}$$

Where W is defined as the sampling dilution rate, and equal to Q/V. Since at $t = 0$, $A_{out} = \tilde{A}_{out}$, from Equation (19), $c = \ln (A_o - \tilde{A}_{out})$. Substituting the value of $c = \ln (A_o - \tilde{A}_{out})$ back to Equation (19) and rearranging,

$$A_{out} = A_o - \left[A_o - \tilde{A}_{out}\right] e^{-Wt} \tag{20}$$

Subtracting and then dividing both sides of Equation (20) by $\tilde{A}_{out}$,

$$\frac{A_{out} - \tilde{A}_{out}}{\tilde{A}_{out}} \times 100\% = E\,\% = \left(\frac{A_o}{\tilde{A}_{out}} - 1\right)\left(1 - e^{-Wt}\right) \times 100\% \qquad (21)$$

Where E is the error in estimating the steady state substrate concentration.

If; for example

$A_o = 2.5$ mg/l

$\tilde{A}_{out} = 0.1$ mg/l

$Q = 1.0$ ml/sec

$V = 100$ ml

$t = 2$ sec.

are substituted into Equation (21), the error is 47.5% indicating how important it is to consider the influence of the fresh medium introduced into a sample during the sampling procedure. In this work, no fresh medium was allowed to enter the reactor during sampling, so this sort of error was avoided.

III. Error induced by cells growing on the reactor wall.

The problem of microorganisms adhering to the walls of the continuous culture reactor is frequently cited (Herbert et al., 1956). This problem may become serious in cases where the culture volume is

small and the cell population is very low. The following derivations show the influence on kinetics study of cells growing on the reactor wall. Owing to the cell growth on the reactor wall, the mass balance of cells in the reactor (Equation 2) has to be modified as follows:

$$V \frac{dX}{dt} = - F X + V\mu X + V \left(\frac{dX}{dt}\right)_w \tag{22}$$

where $\left(\frac{dX}{dt}\right)_w$ is the release rate of cells from the reactor wall. The other terms are defined as before. At steady state, $\frac{dX}{dt} = 0$, and Equation (22) becomes

$$r = \mu + \frac{1}{X} \left(\frac{dX}{dt}\right)_w \tag{23}$$

If the release rate of cells from the reactor wall is negligible, Equation (23) is simplified to Equation (4). Equation (23) indicates that due to the effect of cell growth on the reactor wall, the dilution rate is no longer equal to the specific growth rate, μ, of the suspended cells in the reactor.

Similarly, the mass balance of the limiting substrate in the reactor can be written as follows:

$$V \left(\frac{dA_{out}}{dt}\right) = F \left(A_o - A_{out}\right) - \frac{V\mu X}{Y_{aA}} - V \left(\frac{dA_{out}}{dt}\right)_w \tag{24}$$

where $(dA_{out}/dt)_w$ is the rate of consumption of substrate by cells growing on the reactor wall. At steady state, $dA_{out}/dt = 0$, Equation (24) becomes

$$r(A_o - \tilde{A}_{out}) = \frac{\mu\tilde{X}}{Y_{aA}} + \left[\frac{d\tilde{A}_{out}}{dt}\right]_w \qquad (25)$$

Rearranging Equation (25),

$$\tilde{A}_{out} = A_o - \frac{\mu\tilde{X}}{rY_{aA}} - \frac{1}{r}\left[\frac{d\tilde{A}_{out}}{dt}\right]_w \qquad (26)$$

If the utilization rate of limiting substrate by the cells growing on the walls is negligible, Equation (26) becomes Equation (9) with $r = \mu$. Equation (26) shows that the steady state limiting substrate concentration, $\tilde{A}_{out}$, depends not only on the rate of substrate utilization by the suspended cells, but also on the rate of consumption by the cells on the walls.

A technique based on the rate of isotope depletion was employed to estimate the utilization rate of limiting substrate by the cells on the wall as well as the total cell mass on the wall under steady state conditions. After two to three weeks of steady state operation the magnetic stirrer was stopped and the contents of the reactor drained through sterilized rubber tubing; the reactor was filled to the previous volume with the same fresh ^{14}C-substrate medium used for the original experiment. Then the magnetic stirrer was started, samples were with-

drawn from the reactor at a series of time intervals for one hour. Samples were filtered immediately through a 0.45 μm membrane filter, and one ml of the filtrate was pipetted into a glass scintillation vial containing one drop of 6N HCl (to remove CO_2). It was shaken for 10 minutes, then 10 ml of the scintillation fluid was added and the ^{14}C-content estimated. The cell mass was determined by the ^{14}C-method (see analytical techniques) after removing the cells from the reactor wall with a rubber policeman. Any depletion of ^{14}C-limiting substrate in the experiment is due to the utilization by cells growing on the reactor wall. Figure 4, a typical experiment, shows that the amount of ^{14}C-limiting substrates (glucose plus mixed amino acids) utilized during the one hour experiment was insignificant. The cell mass on the reactor wall was between 0.4 and 3.6% of the total cell mass in the reactor after two to three weeks of operation. Consequently, in this work, the film of cells on the reactor wall was assumed to have a negligible effect on the intercellular limiting substrate concentration.

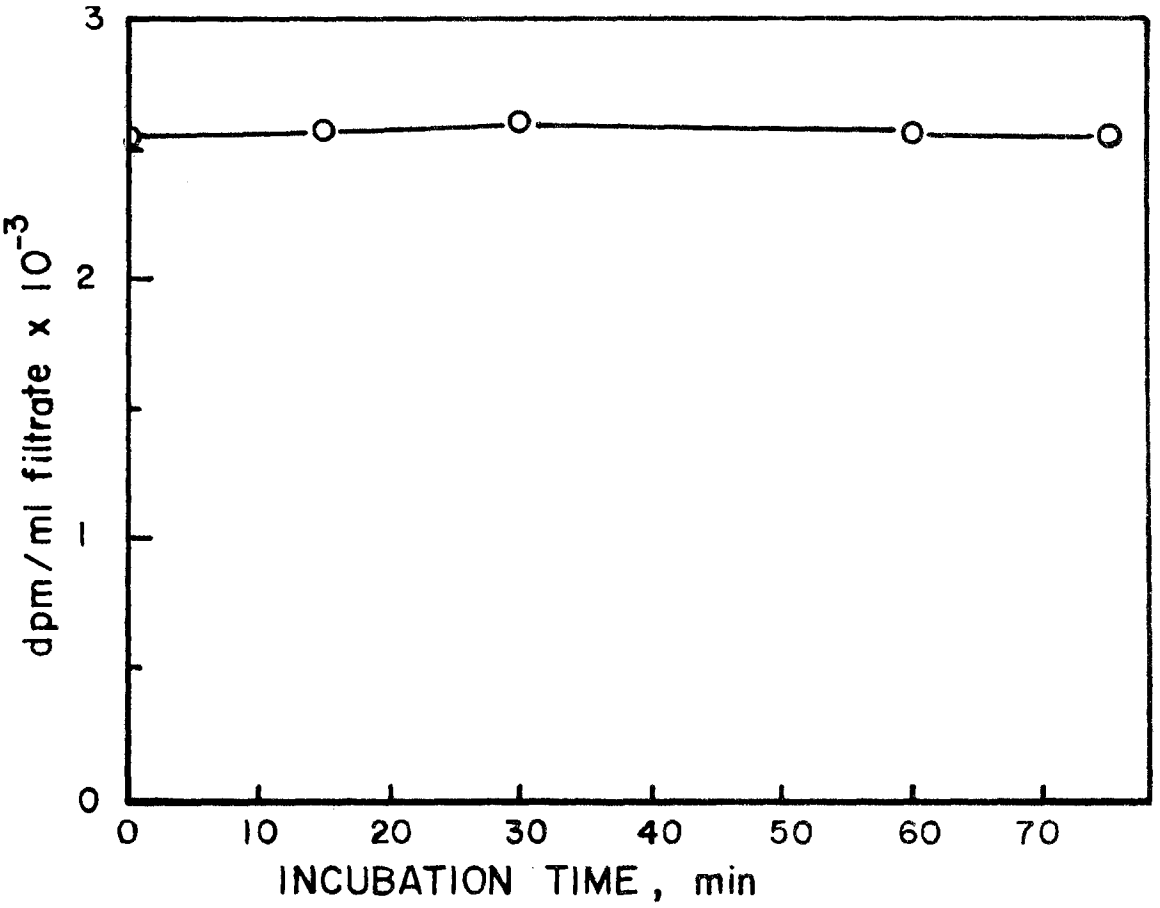

Figure 4. Test for limiting substrate utilization by the cells growing on the reactor wall.

Analytical Techniques

A. Cell mass (dry) estimation

Owing to the tendency of isolate 198 to clump at high cell populations (>10^6 cells/ml), the standard microbiological methods of viable colony counting and electronic particle counting could not be used to estimate cell mass.

The carbon content of a bacterium is equal to about 50% of the cell's dry weight (Luria, 1960), and according to Powell (1967) more than 99% of the bacterial cells at steady state in continuous culture remain viable through a wide range of dilution rates. This information was used to determine cell mass when ^{14}C-labeled limiting substrate was used. One to ten ml of the culture were filtered through a 0.45 µm membrane filter (13 mm diameter) which had been soaked in phosphate buffer (0.2 M, pH 7.0) for five minutes to lessen the amount of arginine or glutamic acid adsorption onto the filter. Ten ml of a 1% saline solution (pH 7) were then passed through the filter to remove any ^{14}C-organic substrate adsorbed onto the filter. The washed filter and collected organisms were immediately blotted by a piece of paper towel and transferred to a glass scintillation vial (20 ml, Kimble) to which ten ml of scintillation fluid were then added. A Beckman LS-100 scintillation system was used for counting, and the counting efficiency was corrected by using a ^{14}C-quenched standards set (Catalog No. 180060; Amersham Searle Corp., Arlington Heights, Illinois).

By assuming that the carbon content of isolate 198 is 50% of its dry weight, and that all the cells in the reactor were viable, the cell mass was estimated. In the fresh medium of a typical [U-^{14}C]arginine limited system, the concentration and radioactivity of arginine was 2.48 mg/l and 1857 dpm per ml of medium respectively. At steady state ($r = 0.246\ hr^{-1}$), the radioactivity of the cell per ml of culture was 426 dpm. The cell mass (dry) was estimated from the following equation:

$$\text{Cell mass} = \frac{(dpm)_c}{(dpm)_{A_o}} \times A_o \times C_A \times M_c \qquad (27)$$

where:

$(dpm)_{A_o}$ = disintegrations per minute of ^{14}C-substrate per ml of fresh medium.

$(dpm)_c$ = disintegrations per minute of ^{14}C-content of the cell per ml of culture.

A_o = concentration of the limiting organic substrate, mg/l, in the fresh medium.

C_A = carbon content of the substrate.

M_c = a factor accounts for the assumption that 50% of the cell material (dry) is carbon (2).

Substituting the above data into Equation (27), the cell mass was 0.49 mg/l.

B. Adenosine triphosphate (ATP) analysis

A method was designed to determine ATP concentration in the range of $1\text{-}25\times10^{-10}$ g ATP per ml of sample solution. Measurements were based on the firefly luciferase catalyzed bioluminescence method of Seliger and McElroy (1960) as described by Holm-Hansen and Booth (1966). Resulting light was detected with a liquid scintillation measurement system (Beckman Beta-Mate II, gain 35%, discriminator 0-100) on single photo cell mode (Schram et al., 1971).

According to McElroy et al. (1967), firefly luciferase (E) catalyzes the formation of luciferyl-adenylate (LH_2-AMP) from luciferin (LH_2) and ATP. The enzyme-bound LH_2-AMP reacts rapidly with oxygen, giving rise to an excited state intermediate which subsequently emits light. One light quantum is emitted for each luciferin molecule oxidized and one molecule of oxygen is used in the process. The reactions catalyzed by luciferase are as follows:

$$LH_2 + ATP + E \overset{Mg^{++}}{\rightleftharpoons} E\cdot LH_2\cdot AMP + PP$$

$$E\cdot LH_2\cdot AMP + O_2 \longrightarrow Light + Products$$

The effects of AMP, ADP, NaCl and Mg^{++} on the ATP measurement have been evaluated by Holmsen et al. (1966) and Dyke et al. (1969). AMP has no effect on the light emission. ADP causes no significant contribution to light emission, unless its concentration is more than 10

times that of ATP. However, chloride ions at a concentration greater than 200 mM cause a 95% inhibition of the reaction. Mg^{++}, necessary for the reaction, is supplied in the amount of 15 mg of $MgSO_4 \cdot 7H_2O$ per ml of enzyme extract. When EDTA was added to the enzyme extract, no light was produced even when relatively large concentrations of ATP were added.

Enzyme preparation. Lyophilized water extracts of firefly lantents were obtained from Sigma Chemical Co., and stored below -15 C until ready for use. Each vial containing the extract from 50 mg of firefly lantents was rehydrated with 5.0 ml of tris (hydroxymethyl) aminomethane (Tris) hydrochloride buffer (0.02 M,pH 7.75, 100 mg/l $MgSO_4 \cdot 7H_2O$). After standing at room temperature for one hour, the suspension was centrifuged at 500 xg for three minutes (Ivan Sorvall Inc., Norwalk, Connecticut). The supernatant was decanted into a clean, dry test tube and immediately 0.2 ml aliquots were pipetted into glass scintillation vials and stored at temperature below -15 C until ready for use. The enzyme preparation took place a few days prior to the experiment.

ATP analytical procedure. ATP standard solutions were prepared by dissolving 10 to 20 mg of ATP (disodium salt) from equine muscle (Sigma Chemical Co., A3127) in tris buffer (0.02 M, pH 7.75, 100 mg/l $MgSO_4 \cdot 7H_2O$), and diluting to a concentration of 10^{-5} g ATP per ml. This was stored below -15 C for not more than one month, although no significant loss of ATP was observed after a standard had been kept

frozen for two months. Immediately before use, the ATP solution was thawed and diluted with tris buffer to the required concentrations (0 to 25×10^{-10} g ATP/ml). The mixture was incubated in a water bath at 25 C. A scintillation vial containing frozen enzyme was placed in the scintillation spectrometer (single photomultiplier mode, machine background 4×10^{4} cpm). The enzyme thawed slowly and when the reaction background ($2\text{-}5 \times 10^{5}$ counts per 0.1 min) became constant, 1.0 ml of the ATP sample was mixed with the enzyme and a timer started. Addition of ATP and loading the glass vial back into the counter took about five seconds. The light emitted by the reaction was recorded several times between 15 seconds and 30 minutes after the initiation of the reaction. Each counting period was 0.1 minute. Figure 5 shows the rate of light emission with time for the luciferin-luciferase-ATP reaction. The response to tris buffer with no ATP added was used for correction of the background. The accumulated counts at different time intervals between 0.5 to 8 minutes were used to find the appropriate time interval at which the accumulated light emission could be related to the amount of ATP added. Figure 6 shows the amount of ATP added and the accumulated counts from 0.5 to 1.5 minute, 2.0 to 4.0 minute, 2.0 to 5.0 minute, 2.0 to 6.0 minute and 2.0 to 8.0 minute. Accumulated counts between 0.5 to 1.5 minute gave the best linear response, although sensivity was lower than during the other intervals. Luminscence response from the luciferin-luciferase-ATP reaction was integrated over the linear 0.5 to 1.5 minute period. Each of the prepared ATP standard

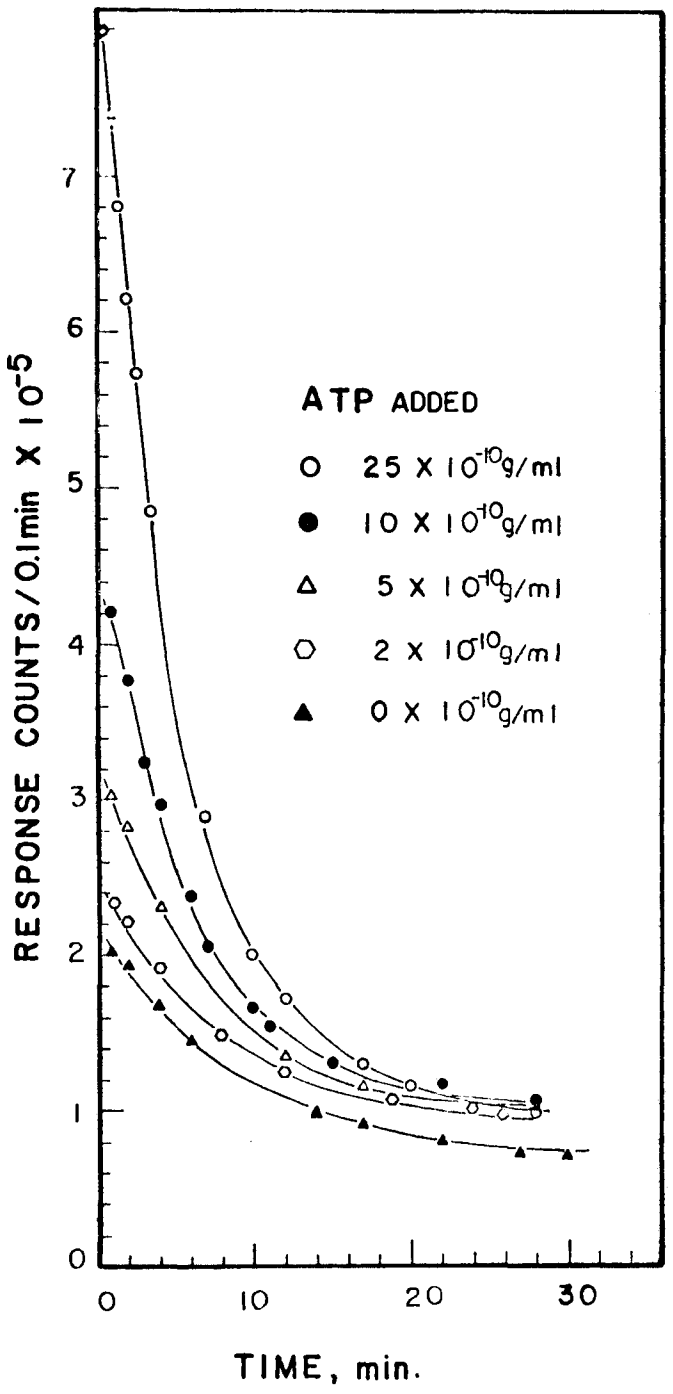

Figure 5. Time course of light emission from the luciferin-luciferase-ATP reaction.

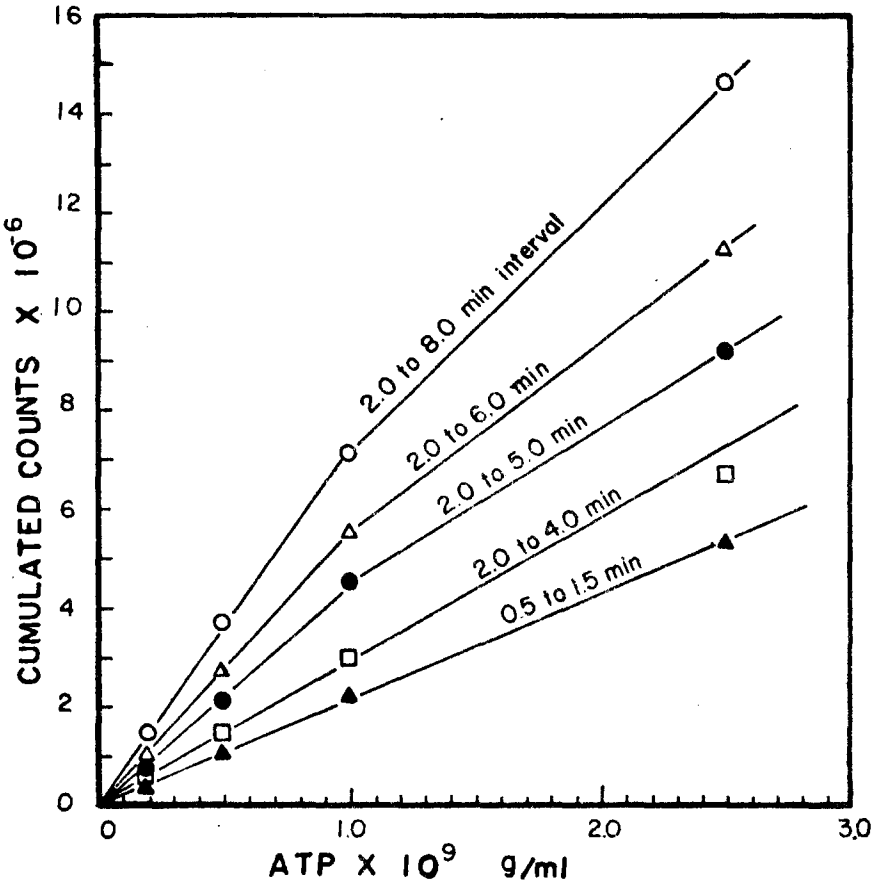

Figure 6. Optimum response interval selection for ATP assay.

solutions was analyzed by the above method. Figure 7 shows a typical standard curve.

Standards were run simultaneously with samples to account for variations in enzyme activity. The response due to slightly different amounts of enzyme in each vial introduced by errors in pipetting, was determined by adding tris buffer to several vials which had different enzyme activity. Figure 8 shows these results which suggest that if, for example, the background activity of the enzyme were 3.5×10^5 counts/0.1 min the background correction for the sample would be 0.93×10^6 counts.

C. Enzyme based method for glucose analysis

Methods for the assay of glucose are sometimes based on coupling the hydrogen peroxide generated by the action of glucose oxidase with a suitable hydrogen donor to give a chromogenic product. The product is then measured spectrophotometrically (Nelson and Huggins, 1974; Messer and Dahlqvist, 1966). These methods are specific and accurate but they are not sensitive enough for quantitative determinations of glucose concentrations below 1.0 mg/l. Although lower amounts may be estimated by isolation of the glucose from the sample and then concentration prior to enzymatic measurement, the procedure is time consuming, requires rather great volume of sample and suffers from an unknown amount of glucose being lost during the course of isolation and concentration.

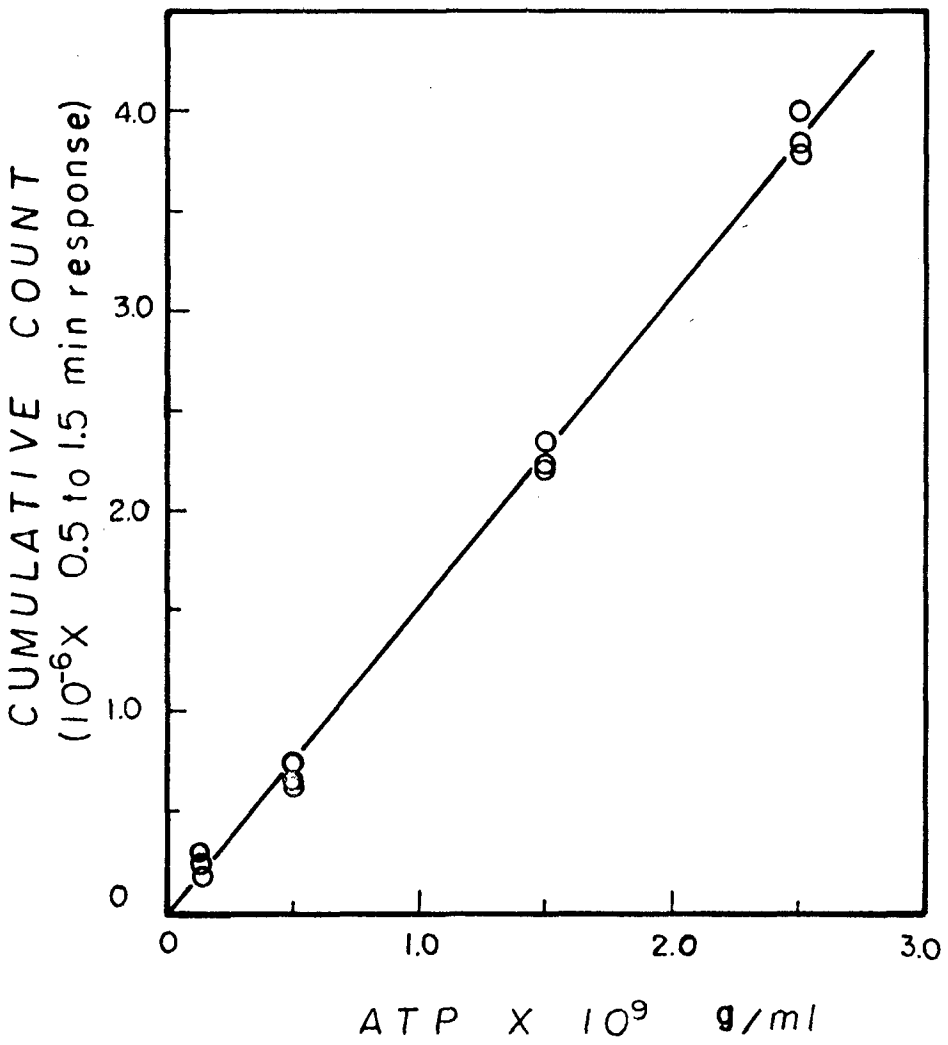

Figure 7. ATP assay standard curve.

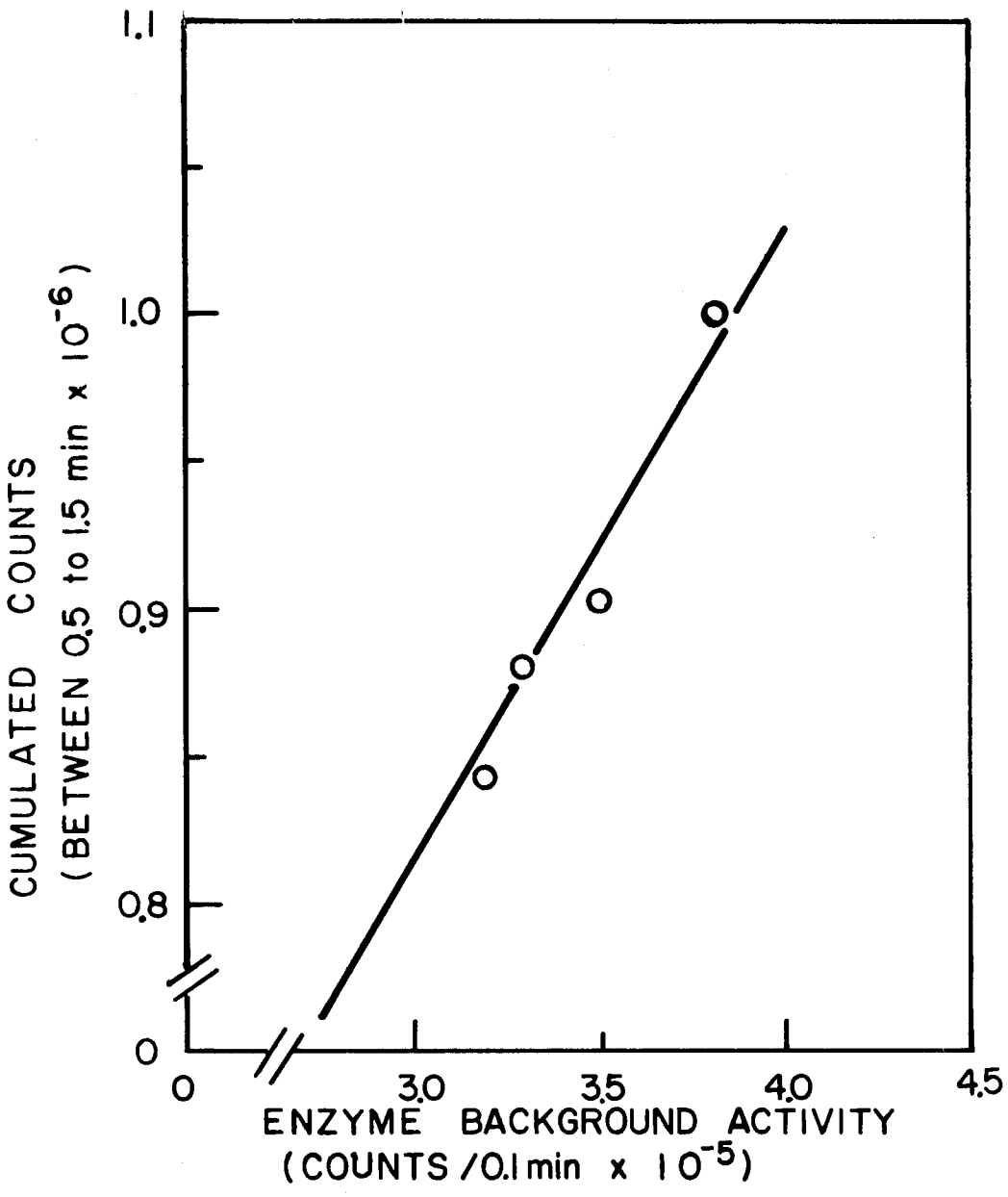

Figure 8. Effect of the enzyme background activity on ATP background correction.

An enzymatic microanalysis of glucose concentration was developed requiring no changes in sample concentration except for the adjustment of pH. This technique involves the utilization of hexokinase in the following reaction:

$$\text{Glucose} + \text{ATP} \xrightarrow[\text{pH } 8.5]{\text{Hexokinase}} \text{ADP} + \text{Glucose-6-Phosphate} + H^{+}$$

The amount of ATP used in the above reaction was estimated by the ATP analysis method described previously. The consumption of ATP was correlated with the amount of glucose added. Before the hexokinase extract could be used in this procedure, it was treated with glucose oxidase to remove any contaminating glucose which could result in a measurement error. This analysis was designed for the estimation of glucose concentrations between 0 to 2.5 mg/l with the lowest level of confidence at about 0.05 mg/l. The analysis recovery, by adding glucose directly to the sample, was 88%.

The properties of hexokinase and glucose oxidase have been studied very intensively (McDonald, 1955; Darrow and Colowick, 1962; Kenneth and Colowick, 1961; Bentley, 1955). Hexokinase is most stable at pH 5 and it has a broad range of optimum activity between pH 8 and 9 (McDonald, 1955). Glucose oxidase is most stable between pH 3.5 and 8.0, its optimum pH is 5.6 and its activity is rapidly lost at pH 8 and pH 2 (Bentley, 1955). The Michaelis constants for hexokinase (McDonald, 1955) and for glucose oxidase (Bentley, 1955) are 0.015 mM

(30 C, pH 7.5) and 9.2 mM (20°C, pH 5.6) respectively. The carbohydrate carried over with purified hexokinase was reported to be 0.2 to 0.5×10^{-8} moles of hexose per mg of hexokinase (Darrow and Colowick, 1962) or about 1 µg of hexose per mg of hexokinase. The hexokinase used for this study contained less than 2 µg glucose per mg of the enzyme. The contaiminating glucose was removed by using glucose oxidase at pH 5.0 where hexokinase is most stable. The extent of reaction of glucose with glucose oxidase is greater than 95% within an hour. When the pH is changed to 8.5 after this reaction, glucose oxidase is inactivated while hexokinase reaches its optimal activity. Besides, hexokinase has a higher affinity than that of glucose oxidase for glucose (McDonald, 1955; Bentley, 1955) and the amount of hexokinase used in the enzyme preparation is 10 times that of glucose oxidase. Consequently, the effect of glucose oxidase in subsequent reactions with glucose in the sample was negligible.

Enzyme preparation. Fifteen milligrams (150 IU) of hexokinase were dissolved in 1 ml of phosphate buffer (pH 5.1, 0.145 M) and 1.5 mg (25 IU) of glucose oxidase added to it. The buffered solution was shaken occasionally while incubated at 25°C in a water bath for one hour. Then the volume was adjusted to 15 ml with a tris buffer solution (pH 8.5, 0.02 M, 100 mg/l $MgSO_4 \cdot 7H_2O$). This enzyme preparation was used immediately for the glucose analysis.

During incubation of the reaction mixture for the removal of background glucose other preparations were made for the glucose assay.

Glucose standard solution. Glucose standard solutions were prepared by dissolving 10 mg of dextrose into 10 ml of the mineral salts medium, pH 8.5, and diluting to a final concentration of between 0.01 to 1.0 mg glucose/l.

ATP standard solution. ATP at concentrations of 0.6×10^{-6} and 6×10^{-6} g ATP/ml were prepared from standard stock ATP (10^{-5} g ATP/ml) by diluting it with tris buffer solution (pH 8.5, 0.02 M, 100 mg/l $MgSO_4 \cdot 7H_2O$).

Sample preparation. The pH of 5 ml of the continuous culture sample (0 to 2.5 mg glucose/l) was adjusted to 8.5 with NaOH.

Analytical procedure. Two ml of the sample (or glucose standard solution) were pipetted into a glass scintillation vial. A control vial contained 2.0 ml of the inorganic medium. One ml of hexokinase preparation and 1.0 ml of ATP standard solution[1] was then added to each vial (total volume 4.0 ml). One ml solution was removed from the control immediately, and diluted with tris buffer (pH 7.75, 0.02 M, 100 mg/l $MgSO_4 \cdot 7H_2O$) to the range of 1 to 25×10^{-10} g ATP/ml. This was for ATP standard curve preparation (see analytical techniques). Both sample and control vials with remaining reactants were shaken for one hour[2] at room temperature for glucose dependent ATP hydrolysis. One ml each of sample and control was then diluted with tris buffer (pH

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[1] When glucose concentration was between 0.1-10 mg/l, 6×10^{-6} g ATP/ml ATP standard solution was used, while 0.6×10^{-6} g ATP/ml ATP standard solution was used when glucose concentration was below 0.1 mg/l.

[2] No significant difference in the amount of ATP present in the vial was observed between one and two hours of reaction.

7.75, 0.02 M, 100 mg/l $MgSO_4 \cdot 7H_2O$) to an ATP concentration range of 5 to 25×10^{-10} g ATP/ml. The ATP content was then estimated as described previously. The difference between the amount of ATP present in the control and in the sample reflects the degree of ATP utilization and hence the concentration of glucose added to the hexokinase reaction mixture. Figure 9 shows the glucose standard curve prepared by the above technique. The lowest sensitivity of this technique is about 50 µg glucose/l. The reaction went to 75% completion. The same reaction extent has been reported by Kunitz and McDonald (1946) when the hexokinase activity was measured by titrating the amount of acid produced in 30 minutes of reaction. At room temperature, an equilibrium mixture of glucose consists of one-third of α-D-glucose and two-third of β-D-glucose in aqueous solution. The possibility that the hexokinase preparation catalyzed only β-D-glucose and resulted in the apparent incompleted reaction, was investigated: the same extent of reaction was found when α-D-glucose, β-D-glucose or the equilibrium mixture of glucose was treated with the hexokinase.

Measurements were carried out within 30 minutes after dissolving the glucose to avoid isomer interconversion.

Of the hexoses, yeast hexokinase is known to catalyze the phosphorylation of D-fructose and D-mannose. It also catalyzes the conversion of D-glucosamine to D-glucosamine 6-phosphate, and 2-deoxy-D-glucose to 2-deoxy-D-glucose 6-phosphate (McDonald, 1955). Two potential difficulties with the hexokinase assay were investigated;

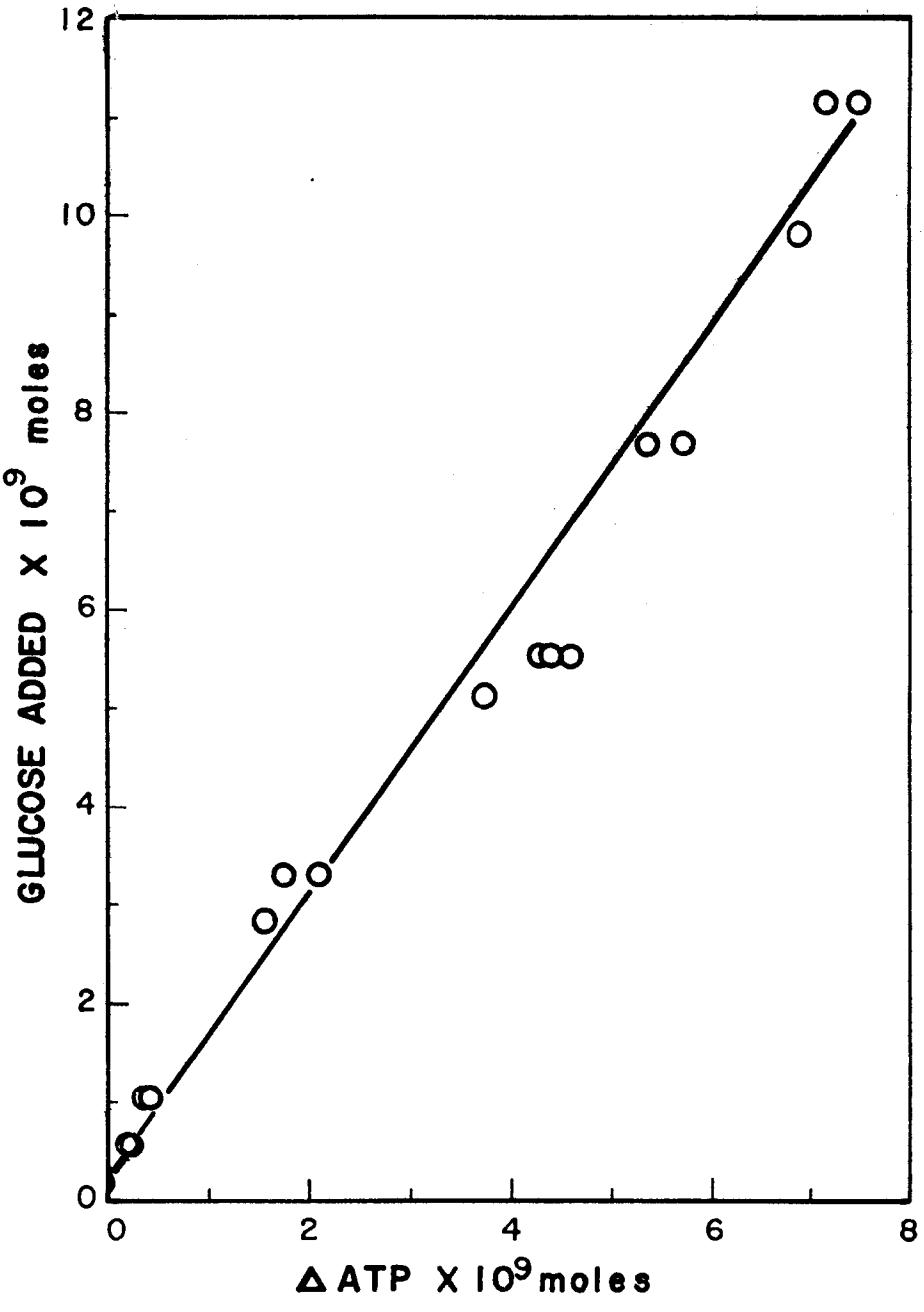


Figure 9. Glucose standard curve.

ATP contamination of the sample from the culture; and hexokinase labile non-glucose substrate contamination of the sample. ATP contamination was tested by comparing hexokinase free sample mixture ATP assay results with buffer controls. No difference was found. The metabolic product influence test made use of the fact that glucose oxidase is specific for glucose oxidation. Since the samples were ATP free, hexokinase based added ATP analyses on glucose oxidase treated samples give the extent of non-glucose dependent hexokinase catalyzed ATP hydrolysis. None was found; buffer solution and glucose oxidase treated samples yielded identical ATP recoveries.

D. Arginine analysis

Amino acids are generally quantitated by means of an amino acid autoanalyzer (Moore et al., 1958; Benson and Patterson, 1965), or thin layer chromatography (Brenner et al., 1965) in which the lowest range of detection is around 0.1-0.5 μg. Fermentation media and biological fluids require desalting and concentration when analyzed by these techniques. The process is time consuming and requires a large quantity of medium; moreover, the recovery is usually low and uncertain. Consequently, a method using [U-^{14}C]arginine and involving no sample concentration was developed to measure the arginine content of a medium. The procedure involved the use of arginase and urease which catalyzed the following reactions (Greenberg, 1960; Varner, 1960):

$$[U\text{-}^{14}C]\text{arginine} + H_2O \xrightarrow[\text{pH } 9.5]{\text{Arginase}} [U\text{-}^{14}C]\text{orithine} + [^{14}C]\text{urea}$$

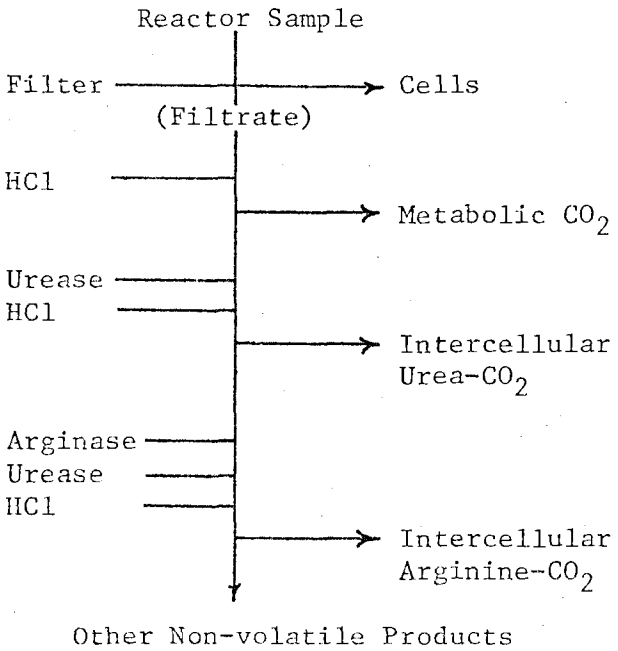
$$[^{14}C]\text{urea} + H_2O \xrightarrow[\text{pH } 7.0]{\text{Urease}} [^{14}C]\text{carbon dioxide} + 2NH_3$$

The $[^{14}C]$carbon dioxide was collected in a 30% NaOH solution.

Enzyme preparation. Fifty units of arginase were dissolved in 0.5 ml of phosphate buffer (pH 9.5, 0.01 M). Fifty units of urease were dissolved in 3.0 ml of phosphate buffer (pH 7.0, 0.15 M).

Sample preparation and experimental procedure

When $[U\text{-}^{14}C]$arginine was used as a limiting substrate, the sequence for the analysis of intercellular arginine concentration is as outlined and described below.



The metabolic $^{14}CO_2$ in the acidified sample (5-10 ml) was removed by using the vaccum chamber of a rotary evaporator (Buchler Instruments, Fort Lee, New Jersey) at room temperature. The operation took about 30 sec and the volume lost due to degassing was less than 1%. Three ml of the treated sample were distributed into each vial and its pH was adjusted to 7.0 with NaOH and then 50 units of urease (powder form) were added to the vial. The vial was shaken for two hours at room temperature. After adding one or two drops of 6N HCl, the vial was incubated for another five minutes with occasional shaking and with air blowing over it. This would remove the $^{14}CO_2$ derived from urea in the sample. Then after adjusting the pH to 9.5, the sample was immersed in hot water (95-100 C) for two minutes to denature the enzymes and then cooled to room temperature. After this treatment the sample was introduced into the side wall of a 50 ml conical flask having a 1" high center wall. A 3/4"x3/4" filter paper (No. 589 Carl Schleider and Schuell Co., Keene, New Hampshire) was put into the center wall along with 0.15 ml of 30% NaOH. The arginase preparation (0.5 ml) was added to the sample, the flask was sealed with a rubber stopper and incubated with shaking for two hours[1] at room temperature. The rubber stopper was displaced slightly and 3.0 ml of the urease preparation was injected into the flask which was then sealed quickly. After two hours incubation, one ml of 6N HCl was injected into the reaction

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[1] No $^{14}CO_2$ evolved from the sample during this reaction, indicating that no urease contamination was present in the arginase.

mixture. The flask was again incubated for 20-24 hours at room temperature, the filter paper removed carefully with a forceps and the residue collected in a long nose one ml glass pipet. The well was rinsed four to five times with scintillation fluid. The filter paper, the residue and the scintillation solution rinse were put into a glass scintillation vial and the volume adjusted to 15 ml with additional scintillation fluid. The ^{14}C-content was estimated from liquid scintillation data a few days later. A control containing the same amount of sample but no added enzymes was run simultaneously with the samples. No significant $^{14}CO_2$ appeared in the controls.

Contamination of the urease and the arginase preparations by amino acid decarboxylases was investigated; no significant $^{14}CO_2$ was obtained when [U-^{14}C]amino acid mixtures were treated with urease at pH 7.0 and arginase at pH 9.5. Therefore, this enzyme based arginine analysis is suitable for estimating arginine concentration in mixed amino acid solution.

Arginine standard curve. Ten milligrams of L-arginine were dissolved in 10.0 ml of a medium[1] in which a culture had already been grown; the concentration of arginine was then reduced to 1.0 mg/l by dilution with the culture grown medium, after which 1 µCi/l of [U-^{14}C] arginine was added. The resulting labeled arginine solution was further diluted to produce several solutions having a range of arginine

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[1]This medium was prepared by growing isolate 198 for 96 hours in batch culture in a medium (pH 7.0) which had 2.5 mg/l of arginine as a sole carbon source. The cells were removed by filtration; the filtrate was adjusted to pH 9.5 and sterilized at 15 psig steam pressure for 15 minutes.

concentrations of from 0.1-1.0 mg/1. Two ml of each were used for arginine analysis.

Figure 10 shows an arginine standard curve prepared by the procedure described. A linear relationship exists between the amount of arginine added and the amount of arginine estimated to be present according to the $^{14}CO_2$ experiments which depend upon the following stoichiometric calculation: one molecule of [U-^{14}C]arginine releases one molecule of [^{14}C]urea, and one molecule of [^{14}C]urea releases one molecule of $^{14}CO_2$. Therefore, by measuring the amount of $^{14}CO_2$ collected one can calculate the unknown concentration of arginine in a sample by using the following equation:

$$\text{Arginine estimated} = \frac{(\text{dpm})_{CO_2} \times N}{(\text{dpm})_{A_o,\,\text{arg}}} \times A_{o,\,\text{arg}} \qquad (28)$$

where:

$(\text{dpm})_{CO_2}$ = disintegrations per minute of $^{14}CO_2$ collected per ml of sample added (calculated from cpm and spectrometer efficiency).

$(\text{dpm})_{A_o,\text{arg}}$ = disintegrations per minute of [U-^{14}C]arginine per ml of fresh medium.

$A_{o,\text{arg}}$ = concentration of arginine (mg/l) in the fresh medium.

N = the ratio between atom carbon per molecule and atom carbon cleaved per molecule.

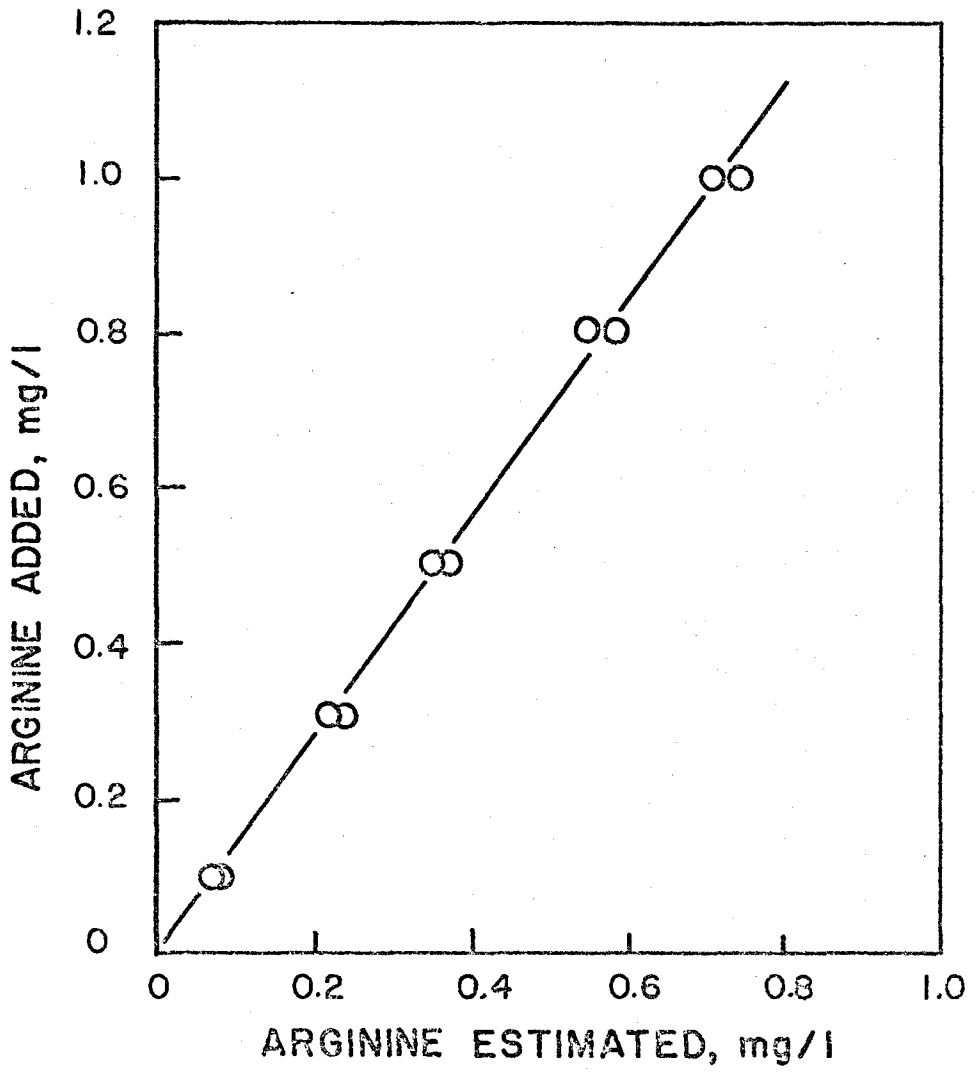

Figure 10. The enzyme based arginine assay standard curve. The arginine concentration was estimated from the amount of $^{14}CO_2$ collected from the urease-arginase reaction.

Equation (28) includes a multiplication factor N of 6 to account for arginine's carbon atoms, only one of which is released as $^{14}CO_2$ in the reactions. Only 75% of the $^{14}CO_2$ radioactivity was recovered from the reactions. The reason for the incomplete reactions is not known. The possibility of reaction inhibition by the metabolic products present in the prepared medium was studied by replacing the prepared medium with the fresh mineral medium; however, no inhibition effects were found, the same percentage of $^{14}CO_2$ radioactivity was recovered.

E. Urea analysis

[^{14}C]urea was diluted with isolate 198 grown medium[1], pH 7.0, to 0.1-1.0 mg urea/l. Fifty units of urease were added to 2.0 ml of the solution, and the mixture was incubated for two hours at room temperature with shaking. Then 0.1 ml of 6N HCl was added and the sample was evaporated to about 1 ml using a glass wool filtered air stream directed over the surface of the sample. Ten ml of the scintillation fluid was added and the ^{14}C-content of the sample was estimated. A control without added urease was run simultaneously with each standard solution. The amount of $^{14}CO_2$ released by the reaction was taken as the radioactivity difference between the control and the sample. Figure 11 shows the standard curve of urea analysis. The reaction was about 97% complete within two hours. No detectable $^{14}CO_2$ was released

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[1]Same medium used for preparing arginine standard solution.

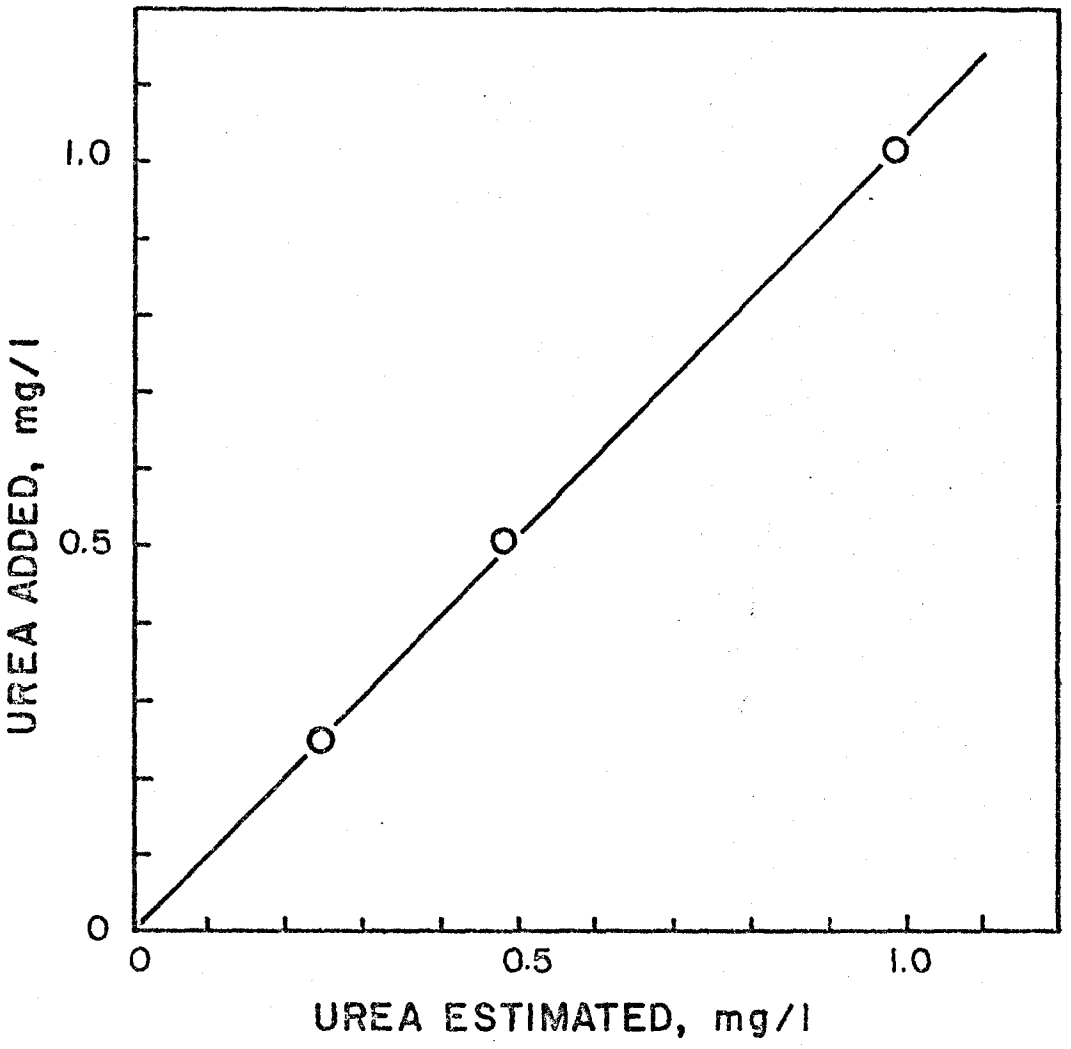

Figure 11. The enzyme based urea assay standard curve. The urea concentration was estimated from the amount of $^{14}CO_2$ collected from the urease reaction.

after two hours. The remaining radioactivity in the sample was subjected to detailed study. The reaction products CO_2 and NH_3 were removed by adjusting the pH of the solution (pH 1 for CO_2, then pH 12 for NH_3) followed by degassing. The solution was adjusted back to pH 7.0 and it was treated with fifty units of urease for two hours. No significant additional $^{14}CO_2$ was released from solution. The radioactivity remaining in the sample is, therefore, due to some other reason than insufficient reactivity, perhaps impurities in the urea supplied.

F. Glutamic acid analysis

Glutamic acid was analyzed using glutamic decarboxylase which catalyzes the removal of the carboxyl group from glutamic acid producing an amine plus CO_2.

$$CH_3(CH_2)_2CHNH_2COOH \xrightarrow[\text{pH 4.6}]{\text{Glutamic decarboxylase}} CH_3(CH_2)_2CH_2NH_2 + CO_2$$

[U-^{14}C]glutamic acid was used and the $^{14}CO_2$ released from the reaction was collected in a 30% NaOH solution as described for arginine analysis. Possible contamination of glutamic decarboxylase with arginine decarboxylase was investigated; if there was any contamination at all the amount was negligible.

Enzyme preparation. Two mg of L-glutamic decarboxylase (1 IU) were dissolved into 2.0 ml of pyridine-pyridoxal buffer, pH 4.6 (0.1 M

pyridine, 6.5×10^{-5} M pyridoxal phosphate). The precipitate was removed by centrifugation at 1000xg for five minutes after standing at room temperature for five minutes. The supernatant was decanted into a clean test tube, and used for the analysis immediately.

Sample preparation and experimental procedure. The metabolic $^{14}CO_2$ in the sample was removed by acidification followed by degassing as described in the arginine analysis procedure. The sample (3 ml) was adjusted to pH 4.6 with NaOH. The reaction was carried out in the conical flask as described previously in the arginine analysis section. Two ml of the enzyme preparation were injected into the outer well containing the sample and the flask sealed with a rubber stopper. Incubation was at room temperature for two hours with shaking. One ml of 6N HCl was subsequently injected directly into the flask to drive off $^{14}CO_2$ and the sample incubated for 20-24 hours at room temperature. The $^{14}CO_2$ content in the center well of the flask was estimated from the radioactivity of the collected NaOH solution.

The concentration of glutamic acid in the sample was estimated according to the following equation:

$$\text{Glutamic acid estimated} = \frac{(\text{dpm})_{CO_2} \times N}{(\text{dpm})_{A_{o,\text{glutamic acid}}}} \times A_{o,\text{glutamic acid}} \quad (29)$$

where:

$(\text{dpm})_{CO_2}$ = disintegrations per minute of $^{14}CO_2$ collected per ml of sample added.

$(dpm)_{A_o,\text{glutamic acid}}$ = disintegrations per minute of [U-^{14}C] glutamic acid per ml of fresh medium.

$A_{o,\text{glutamic acid}}$ = glutamic acid concentration, mg/l, in the fresh medium.

N = the ratio between atom carbon per molecule and atom carbon cleaved per molecule.

Equation (29) includes a multiplication factor N of 5 to account for glutamic acid's carbon atoms, only one of which is released as $^{14}CO_2$ in the reaction.

Glutamic acid standard curve. Ten milligrams of L-glutamic acid were dissolved in 10.0 ml of a medium[1]; the concentration of glutamic acid was then reduced to 1.0 mg/l by dilution with the same medium after which 1 μCi/l of [U-^{14}C]glutamic acid was added. The resulting labeled glutamic acid solution was further diluted to produce several solutions having a range of glutamic acid concentration from 0.1-1.0 mg/l; two ml of each were used for glutamic acid analysis. The glutamic acid standard curve is shown on Figure 12. The extent of reaction was about 70% at completion.

G. Pool extraction and purification

The pool of intracellular metabolites, or simply the "pool material" was extracted with 10% trichloroacetic acid (TCA) at room

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[1]This medium (pH 7.0) contained 1.5 mg/l of glutamic acid and arginine respectively. Isolate 198 had been grown in this medium for 96 hours. The cells were removed by filtration; the filtrate was adjusted to pH 4.6 and sterilized at 15 psig steam pressure for 15 minutes.

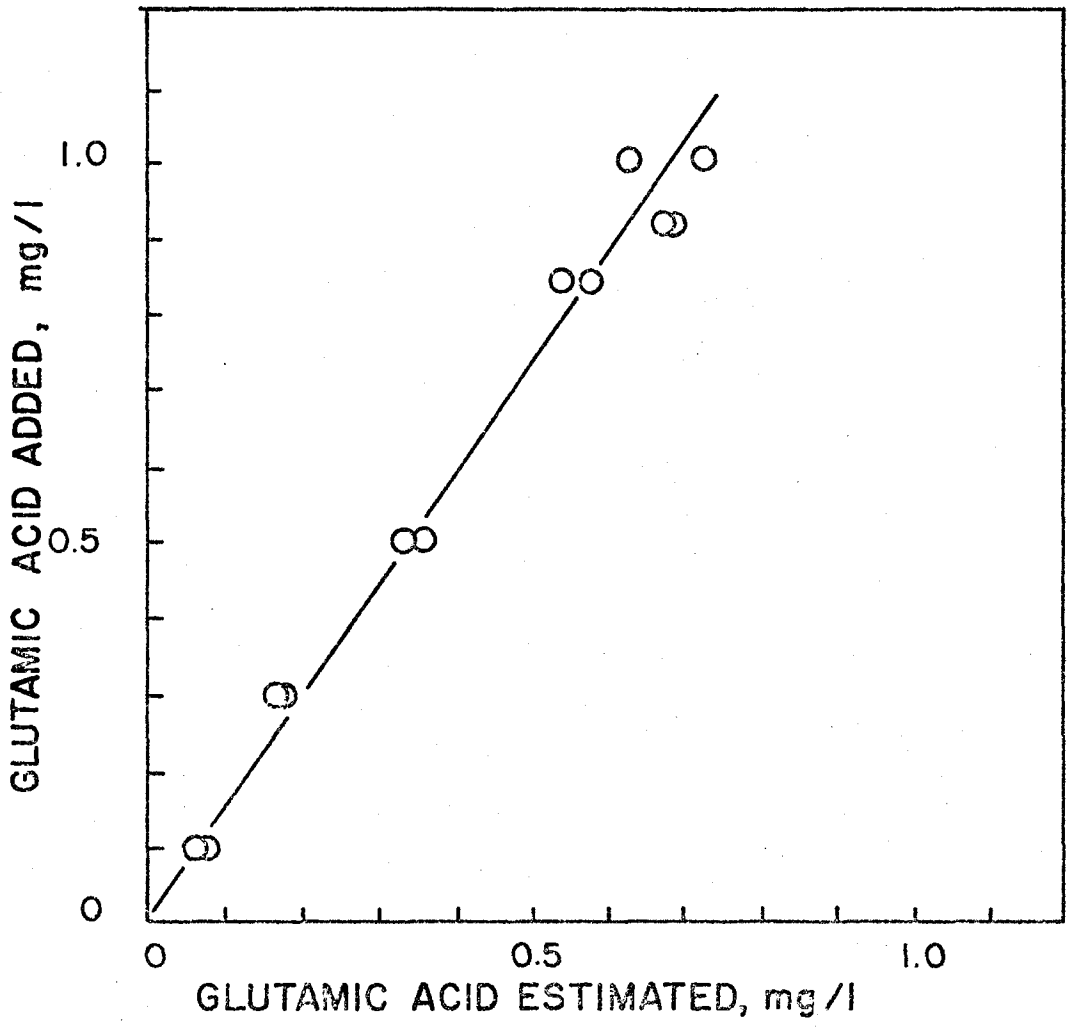

Figure 12. The enzyme based glutamic acid assay standard curve. The glutamic acid concentration was estimated from the amount of $^{14}CO_2$ collected from the glutamic acid decarboxylase reaction.

temperature (Britten & McClure, 1962).

The pool material is defined as the total quantity of low molecular weight intermediates that may be extracted from the cell under conditions such that the macromolecules are not degraded into low molecular weight subunits.

Pool extraction. To harvest the cells, 5-200 ml of culture was filtered through a 0.45 μm membrane filters (13 or 25 mm diameter, depending on volume) treated with phosphate buffer (pH 7.0 0.2 M) for five minutes to prevent the ^{14}C-organic and by-products in the medium from sorbing on it. The filter and retained cells were then washed (10-50 ml of pH 7, 1% saline) to remove sorbed ^{14}C-organic material. Pool extraction was carried out by suspending the washed cells (and filter) in 2-5 ml of cold 10% TCA (depending on volume) and mixing rapidly (vortex mixer, Scientific Industries, Inc., Queens Village, New York) for five minutes. Extracts from this and a second[1] similar procedure containing about 10% of the extractable radioactivity were combined for the total pool extract. For example, 72 ml of a culture, obtained from a [U-^{14}C]arginine limited continuous culture at steady state ($r = 0.204\ hr^{-1}$), containing 39,427 dpm provided a total pool extract of 3,705 dpm. The concentration and radioactivity of [U-^{14}C] arginine in the fresh medium were 2.51 mg/l and 1924 dpm/ml respectively. Since the extract composition of the pool material is unknown,

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[1] A third extraction was not necessary, because no significant amount of pool material was obtained. The first extraction pulled out about 90% of the pool material.

we assume the average pool material is 50% carbon and use the following equation for estimating the amount of pool extract.

$$\text{Pool material} = \frac{(\text{dpm})_p}{(\text{dpm})_{A_o}} \times A_o \times C_A \times M_p \qquad (30)$$

where:

$(\text{dpm})_p$ = disintegrations per minute of ^{14}C-pool extract per ml of culture.

$(\text{dpm})_{A_o}$ = disintegrations per minute of ^{14}C-substrate per ml of the fresh medium.

A_o = concentration of the limiting organic substrate, mg/l, in the fresh medium.

C_A = percentage of carbon content in the substrate.

M_p = a factor accounts for the assumption that 50% of the pool material (dry) is carbon (2).

For mixed substrate studied, A_o, will be the concentration of total limiting substrate, mg/l, in the fresh medium, and C_A will be the average percentage of carbon in the substrate. When equation (30) is divided by equation (27), we obtained equation (31):

$$\tilde{S}_p = \frac{(\text{dpm})_p}{(\text{dpm})_c} \qquad (31)$$

where $\tilde{S}_p$ is defined as grams of pool material per gram of cell mass at steady state. Substituting the values of $(\text{dpm})_p$ and $(\text{dpm})_c$ from the above experimental data into equation (31), we obtained $\tilde{S}_p = 0.094$

which means that there are 0.094 g of intracellular low molecular weight organic intermediates per gram of cell mass when isolate 198 grows at 0.204 hr^{-1} in an arginine limited medium.

Pool purification. Before proceeding with two dimensional thin layer chromatography to identify the pool constituents, it was necessary to remove the high amount of TCA from the pool extract by ether extraction (cold diethyl ether in 2 ml portions). Although the extraction procedure was repeated 8-10 times, only a total 3% of the pool material was lost to the ether phase. This might be due to the removal of pool lipid compounds by ether. One drop of 6N HCl was added to the TCA free pool material which was dried under a glass wool filtered air stream (or N_2) directed over its surface. The dried TCA free pool material was stored below 0 C until it was to be chromatographed.

H. Thin layer chromatography (TLC)

By combining the techniques of TLC and autoradiograph, glutamic acid was identified as the dominant pool constituent as compared to the rest of the pool components present. Therefore, only the glutamic acid and arginine in the pool were identified and quantified. Two dimensional TLC was used.

Fifty µl of a solution containing glutamic acid and arginine (0.1 mg/ml each) were added to the dried TCA free ^{14}C-pool material. Six to eight µl of this mixture were spotted (Microcap-2µl, Analtech,

Canoga Park, California) onto a 20x20 cm sheet of commercial silica gel on fiber glass ITLC type SA (Gelman Instrument Co., Ann Arbor, Michigan). Separation of the amino acids contained in the pool material required a combination of two solvent systems (Brenner et al., 1965). The solvents were prepared just before use and were allowed to ascend until the solvent front was 15 cm above the origin. The first dimension was run with the system composed of chloroform-methanol-17% ammonium hydroxide 40:40:20 (v/v). After its removal from the tank, the sheet was dried in a ventilated hood for 30 minutes. The second dimension development was in a phenol-water 75:25 (w/w) solvent system which had been prepared about one hour before. Seventy-five grams of phenol were mixed with 25 ml of double distilled tap water including 10 mg of Na_2SO_3 to prevent phenol oxidation. A hot water bath was used to facilitate solution, then the solvent cooled to room temperature.

Upon completion of the second dimension, the sheet was dried in an air ventilated hood overnight and colors developed with a ninhydrin spray (Sigma Chemical Co., Missouri). The glutamic acid and arginine spots were cut from the chromatogram, placed in vials with scintillation fluid and counted to determine the amount of ^{14}C present. The recoveries of [U-^{14}C]glutamic acid and [U-^{14}C]arginine in this technique were 95% and 81% respectively when taking 0.5% of the chromatogram for counting.

RESULTS

Batch culture

In batch culture, isolate 198 grew much faster in the present of multiple organic sources than when just one was present. Table 4 shows maximum growth rates (μ_{max}) and lag phases[1] for isolate 198 grown in yeast extract, arginine or glucose medium. In yeast extract medium, the maximum growth rate was five-fold greater than that found in glucose or in arginine medium. However, in continuous culture growth rates in arginine and in glucose medium were substantially higher.

Table 4. Growth rates and lag phases of isolate 198 in batch culture with various organic substrates.

Carbon source	μ_{max} (hr^{-1})	Lag phase (hr)
Yeast extract (10g/l)[2]	0.35	10
Glucose (0.0025g/l)[3]	0.069	50
Arginine (0.0025g/l)[3]	0.07	10

[1]Time between inoculation and growth at the maximum rate.

[2]Growth was followed by measuring the optical density of the culture at 625 mμ.

[3]Growth was followed by measuring the cell mass with the ^{14}C-method described in the analytical section. [U-^{14}C]arginine and [U-^{14}C] glucose were used for the experiment, respectively.

Continuous culture in arginine limiting medium

Arginine, an excellent carbon source for isolate 198 growth in batch culture as shown above, was one of the substrates chosen for the kinetic study of carbon limited metabolism in continuous culture. The range of dilution rates used to set the growth rates during these continuous culture studies was between 0.01 and 0.35 hr^{-1}.

Figure 13 shows the intercellular arginine concentration with respect to the dilution rate at steady state. Clearly, there is a threshold concentration of intercellular arginine as dilution rate approaches zero. The value of the threshold concentration, obtained by extrapolation to zero growth rate, is 0.07 mg/l. The maximum growth rate estimated from Figure 13 was 0.36 hr^{-1}.

The low molecular weight metabolic intermediates or "pool material" provided 9 to 16% of the cell material (g/g cells,dry). The response of pool material concentration to the increase of dilution rate can be seen from Figure 14. The pool material increased slowly from its threshold concentration as dilution rate increased from 0.01 to 0.2 hr^{-1}. When dilution rate was greater than 0.2 hr^{-1}, the pool material increased rapidly with growth rate and reached its maximum value at the maximum dilution rate (maximum growth rate).

An extremely large amount of glutamic acid was contained in the pool material. The pool glutamic acid accounted for up to 39% of the total pool material and 6.5% of the cell dry weight. The threshold concentration of pool glutamic acid was about 35 mg/gm cell dry. Pool

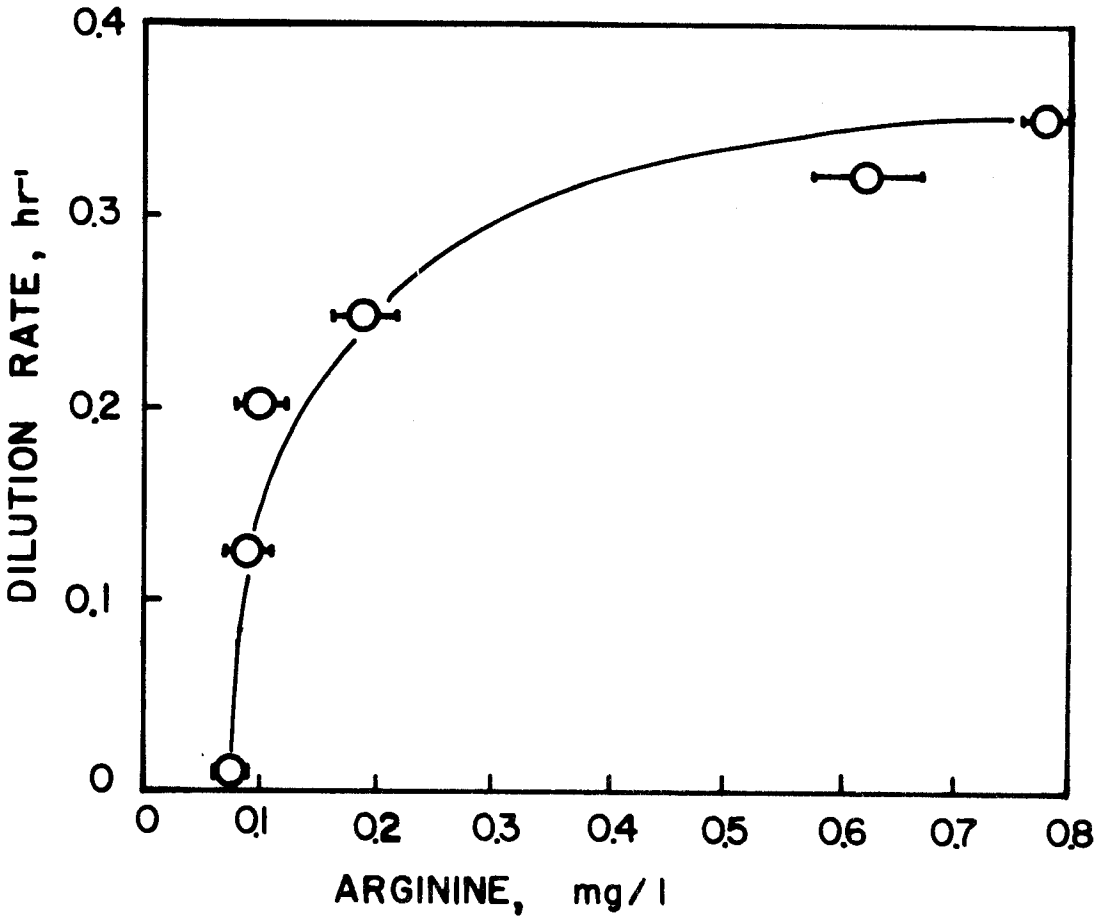

Figure 13. The relationship between steady state arginine concentration and growth rate in arginine limited continuous culture.

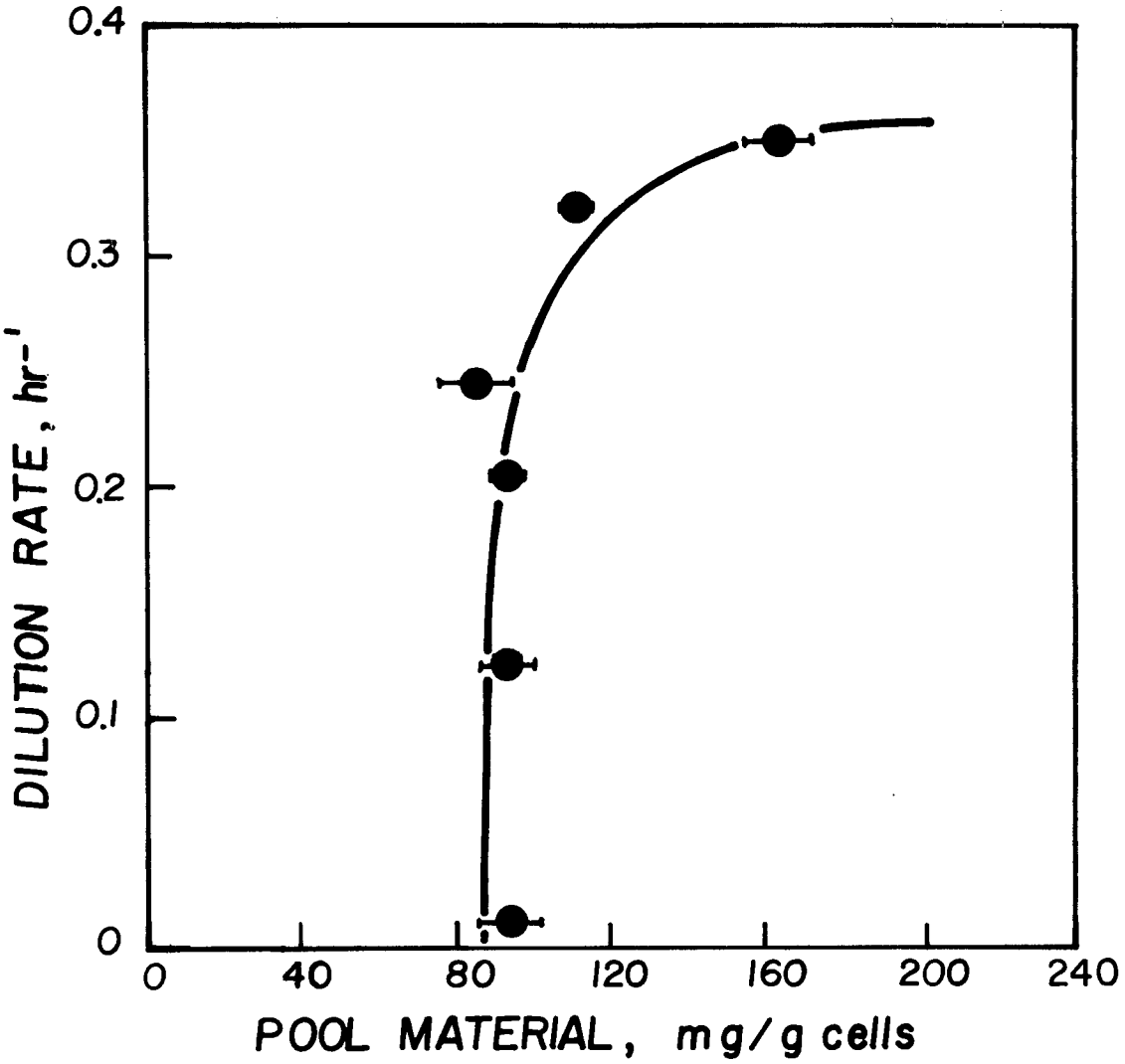

Figure 14. The relationship between growth rate and the TCA extractable intracellular substrate concentration in arginine limited continuous culture.

glutamic acid indeed reflects the growth rate. The relationship between the pool glutamic acid and the dilution rate is shown in Figure 15. Although the cell utilized a large amount of arginine from the medium, there were only trace amounts of arginine found in the pool extracts at all growth rates.

Urea, organic by-product and CO_2 formation as well as cell yield during continuous culture in arginine limiting medium are shown in Figure 16. All the urea derived from arginine was excreted back into the medium at a dilution rate of 0.01 hr^{-1}. However, only half of the arginine derived urea was found in the medium at a higher dilution rate. No significant amount of organic by-product was excreted at either low or high dilution rates. The maximum amount of organic by-product excretion was observed at a dilution rate of 0.2 hr^{-1}; this accounted for 0.65 g/g cells dry. The cell yield was between 0.2 and 0.25 g cells produced/g arginine consumed. The CO_2 formation amounted to about 4 g/g cells dry throughout the experiments.

A typical continuous culture in arginine limiting medium at a low growth rate is shown in Figure 17. Detailed results of the arginine limited growth can be found in Appendix A. The results are summarized in Table 5.

Continuous culture in glucose limiting medium

Glucose was the second limiting organic substrate chosen for this study. The relationship between intercellular glucose concentration

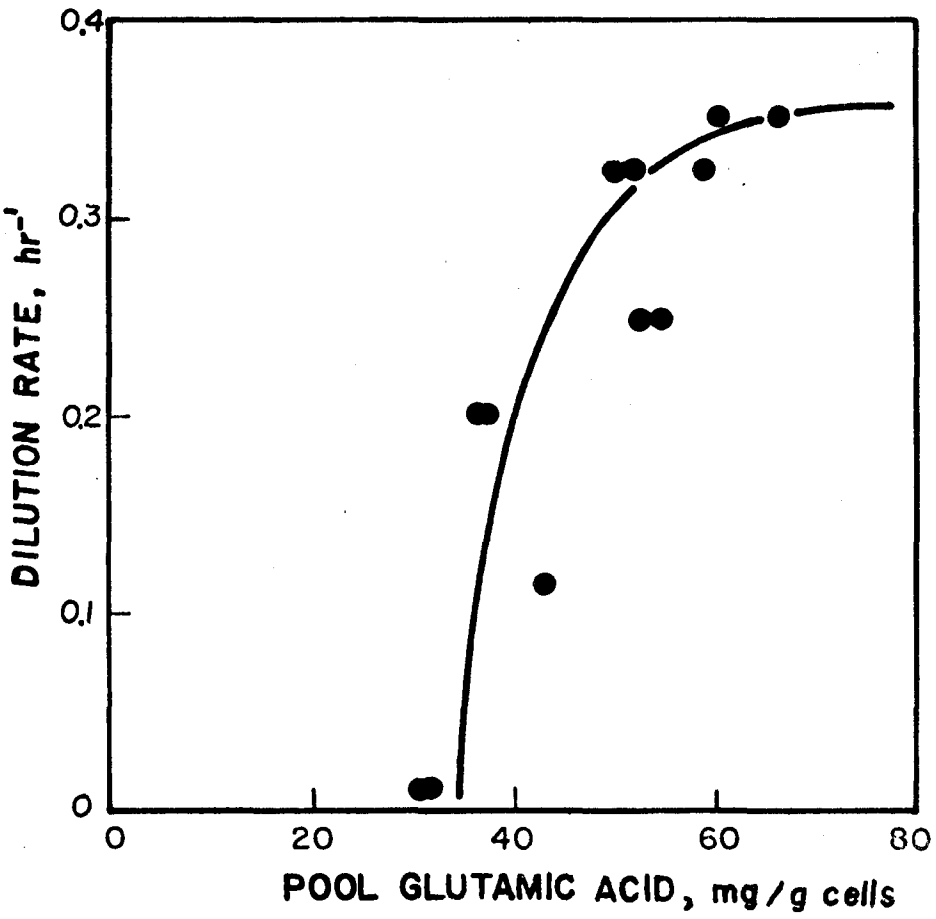

Figure 15. The response of growth rate to the intracellular glutamic acid concentration in arginine limited continuous culture.

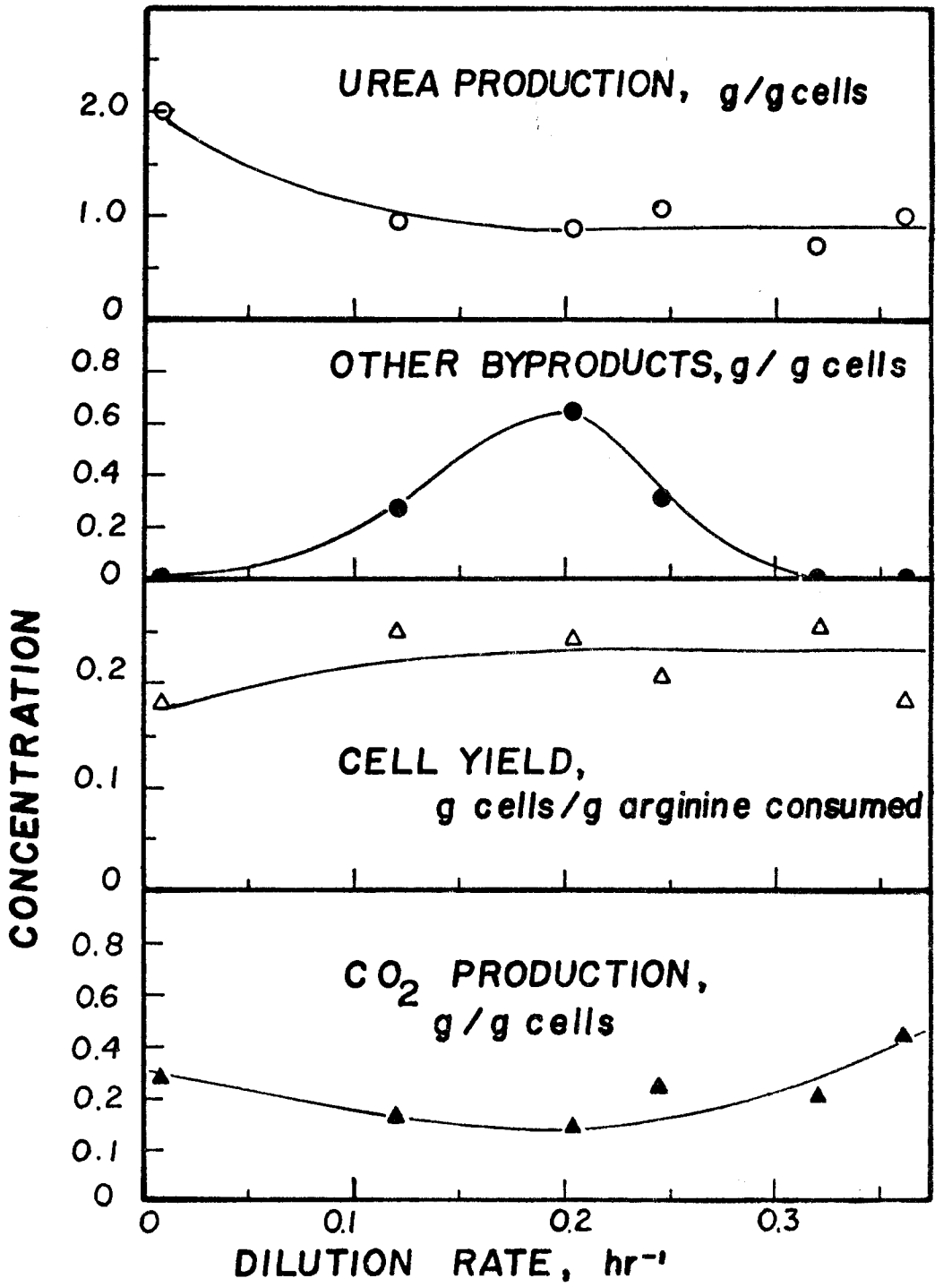

Figure 16. Steady state urea, byproducts, cell yield and CO_2 at various growth rates in arginine limited continuous culture.

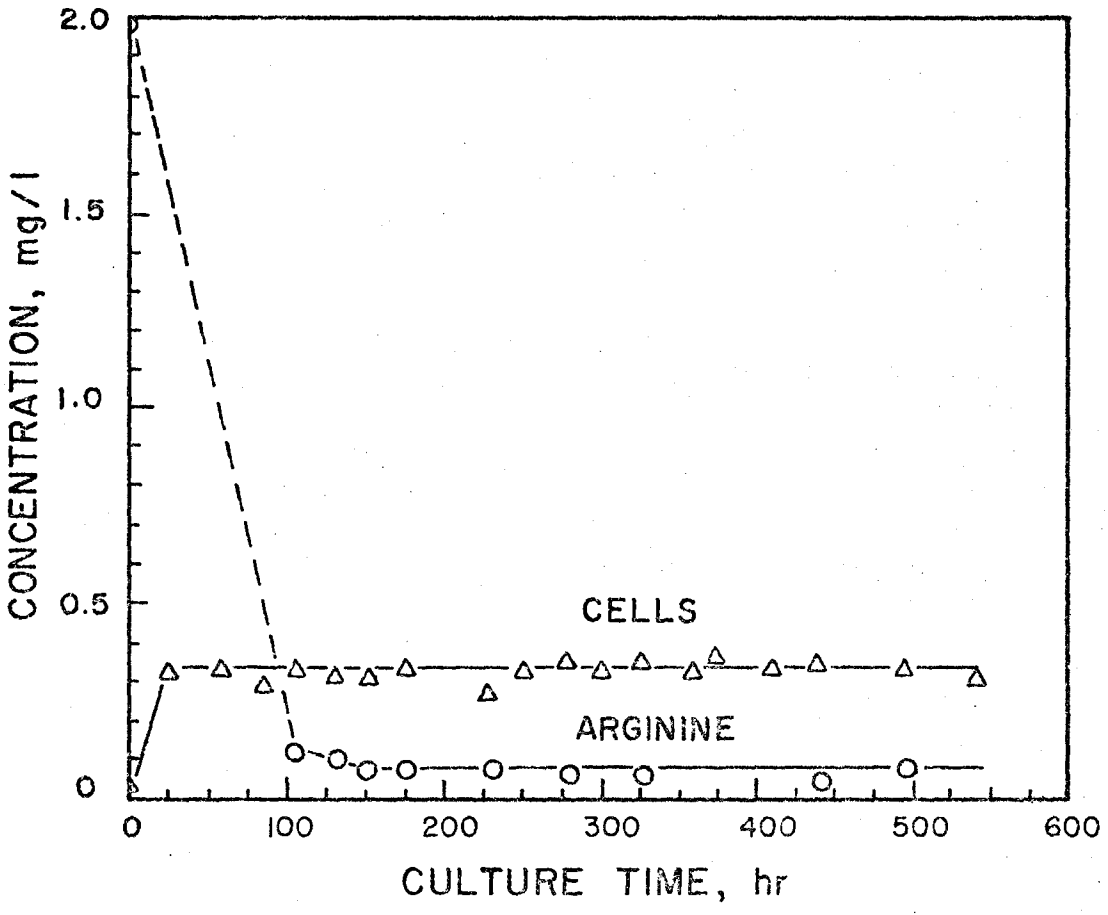

Figure 17. Time course of arginine limited continuous culture ($r=0.01\ hr^{-1}$).

Table 5. Steady state intercellular and intracellular concentrations in arginine limited continuous culture.

Growth rate (hr^{-1})	Intercellular				Intracellular	
	Arginine (mg/l)	Byproduct[2] (g/g cells)	Urea (g/g cells)	CO_2[1] (g/g cells)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.010	0.07 ± 0.01	0	2.0	4.9	97.8 ± 11	32
0.123	0.08 ± 0.02	0.27	0.9	3.9	94.4 ± 6.7	43.7
0.204	0.10 ± 0.02	0.65	0.9	2.9	93.4 ± 3.4	36.6
0.246	0.19 ± 0.03	0.31	1.1	4.7	86.0 ± 10	49.1
0.320	0.63 ± 0.05	0	0.6	4.2	110.0 ± 2.0	54
0.350	0.78 ± 0.02	0	1.0	6.4	163.0 ± 9.0	63.5

[1]The amount of CO_2 was estimated from the difference in ^{14}C content between the fresh medium and the culture medium which had been acidified and degassed.

[2]The amount of organic byproduct excreted was estimated by substracting the tatol amounts of ^{14}C content of the cells, the arginine remained, the urea and the CO_2 from the ^{14}C content of the fresh medium.

and dilution rate is shown in Figure 18. By extrapolating to zero growth rate, a significant threshold concentration of glucose was found; 0.2 mg glucose/l. The maximum growth rate, estimated from Figure 18, is 0.16 hr^{-1} which is two-fold higher than that observed in batch culture.

Although the limiting substrate was glucose, a non-nitrogenous compound, glutamic acid was still the dominant pool constituent which comprised about 35% of the total pool material and 2-5% of the cell material. Figure 19 shows the response of pool material and pool glutamic acid concentration with respect to dilution rate. The range of concentrations for total pool constituents and for pool glutamic acid was 75 to 140 and 24 to 55 mg/g cells respectively. Surprisingly they showed minimum values at intermediate growth velocities and maximum concentrations at low growth velocities rather than increasing in concentrations with growth rate.

Cell yield, organic by-product and CO_2 formation during glucose limited growth are shown in Figure 20. Cell yield data indicate that isolate 198 metabolized glucose more efficiently at very low growth rate. The cell yield was 0.27 (g cells produced/g glucose consumed) at 0.016 hr^{-1} dilution rate. However, the average cell yield at higher dilution rates was only 0.125. The amount of organic by-product and CO_2 formation at very low dilution rate was considerably reduced as compared with those values obtained at higher dilution rates. These observations can account for the higher cell yield at

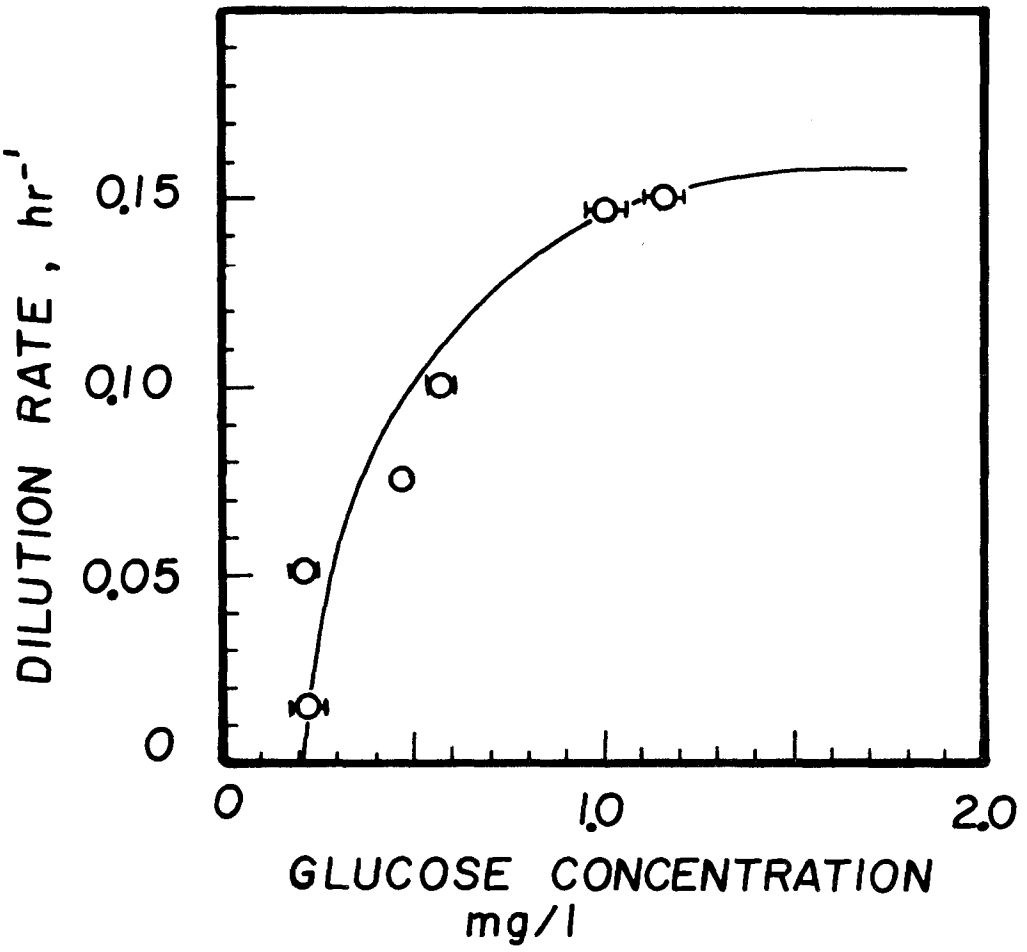

Figure 18. The relationship between steady state glucose concentration and growth rate in glucose limited continuous culture.

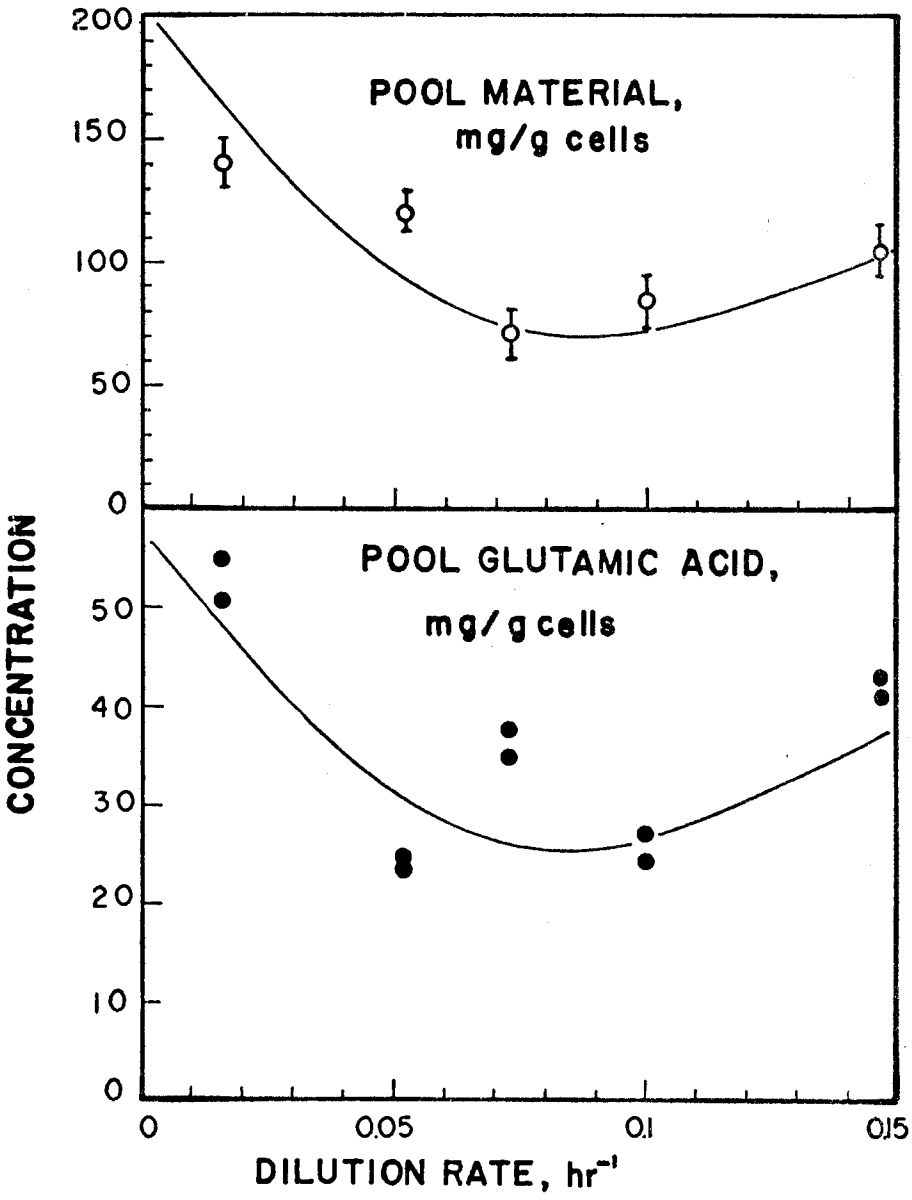

Figure 19. The response of growth rate to the intracellular substrate concentration (above) and the intracellular glutamic acid concentration (below) in glucose limited continuous culture.

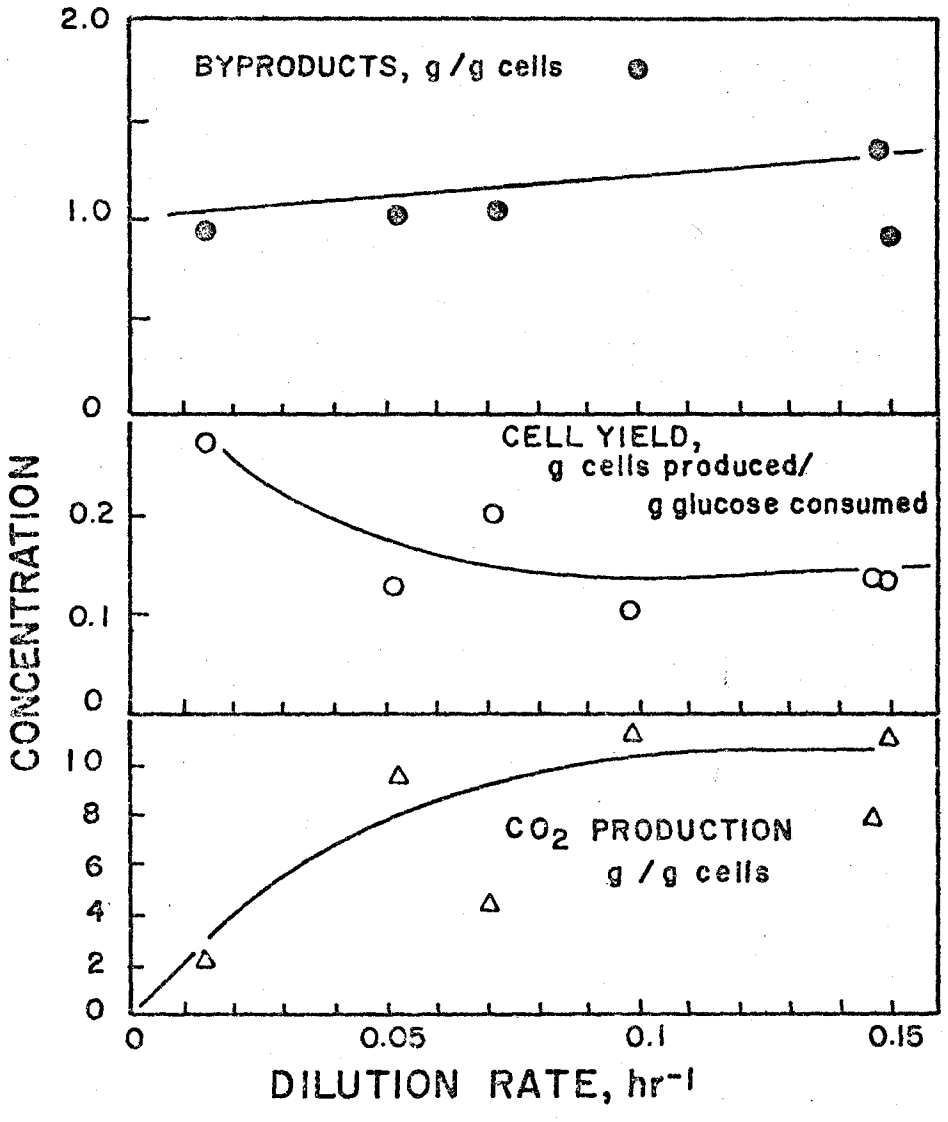

Figure 20. Steady state byproducts, cell yield and CO_2 at various growth rates in glucose limited continuous culture.

very low dilution rates observed.

Figure 21 shows a typical continuous culture of isolate 198 growing in glucose limiting medium at a low growth rate. Detailed results of the glucose limited growth studies can be found in Appendix B and the results are summarized in Table 6.

Continuous culture in arginine-glucose limiting medium

After considering the kinetics of arginine and glucose limited growth, both substrates were combined and the growth kinetics were re-examined. These results are summarized in Figure 22. This figure indicates that arginine and glucose were utilized simultaneously rather than sequentially as had been found for some microorganisms (Monod, 1949). Above a dilution rate of 0.25 hr^{-1}, the intercellular glucose concentration became unstable. When the dilution rate was further increased to 0.35 hr^{-1}, glucose utilization in this carbon limited system no longer occurred.

The steady state values of intercellular arginine and glucose concentration with respect to dilution rate are shown in Figure 23(a). This is followed by similar kinetic curves expressing growth velocities when arginine [Figure 23(b)] and glucose [Figure 23(c)] are present separately, rather than together [see Figure 13 and 18]. It is clearly shown that glucose was utilized more efficiently (i.e. to a lower steady state concentration) in arginine-glucose limiting medium than when it was the sole limiting substrate. The opposite is

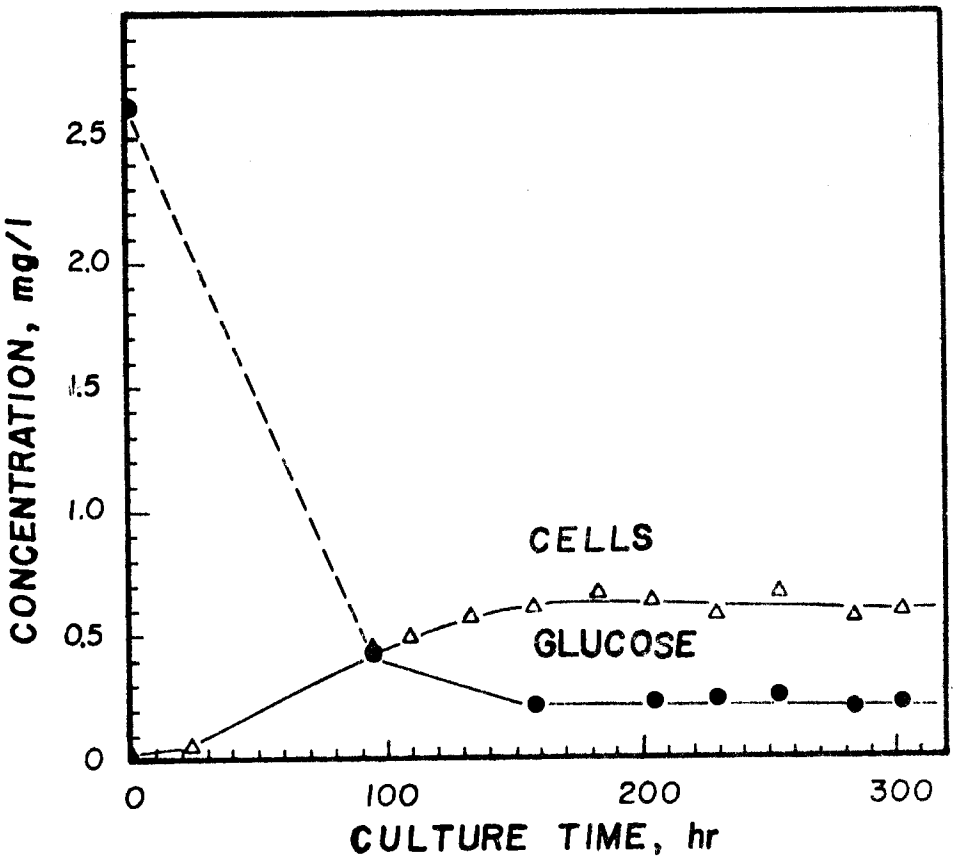

Figure 21. Time course of glucose limited continuous culture ($r=0.016\ hr^{-1}$).

Table 6. Steady state intercellular and intracellular concentrations in glucose limited continuous culture.

Growth rate (hr^{-1})	Intercellular			Intracellular	
	Glucose (mg/l)	Byproducts (g/g cells)	CO_2	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.016	0.22 ± 0.02	0.9	2.6	141 ± 10	53.6
0.052	0.23 ± 0.02	1.1	9.7	120	24.0
0.073	0.50	1.0	4.0	72 ± 13	37.0
0.100	0.60 ± 0.01	1.8	11.2	85 ± 11	25.5
0.148	1.01 ± 0.06	1.4	7.8	105 ± 13	42.2
0.150	1.17 ± 0.05	0.8	11.1	--	--

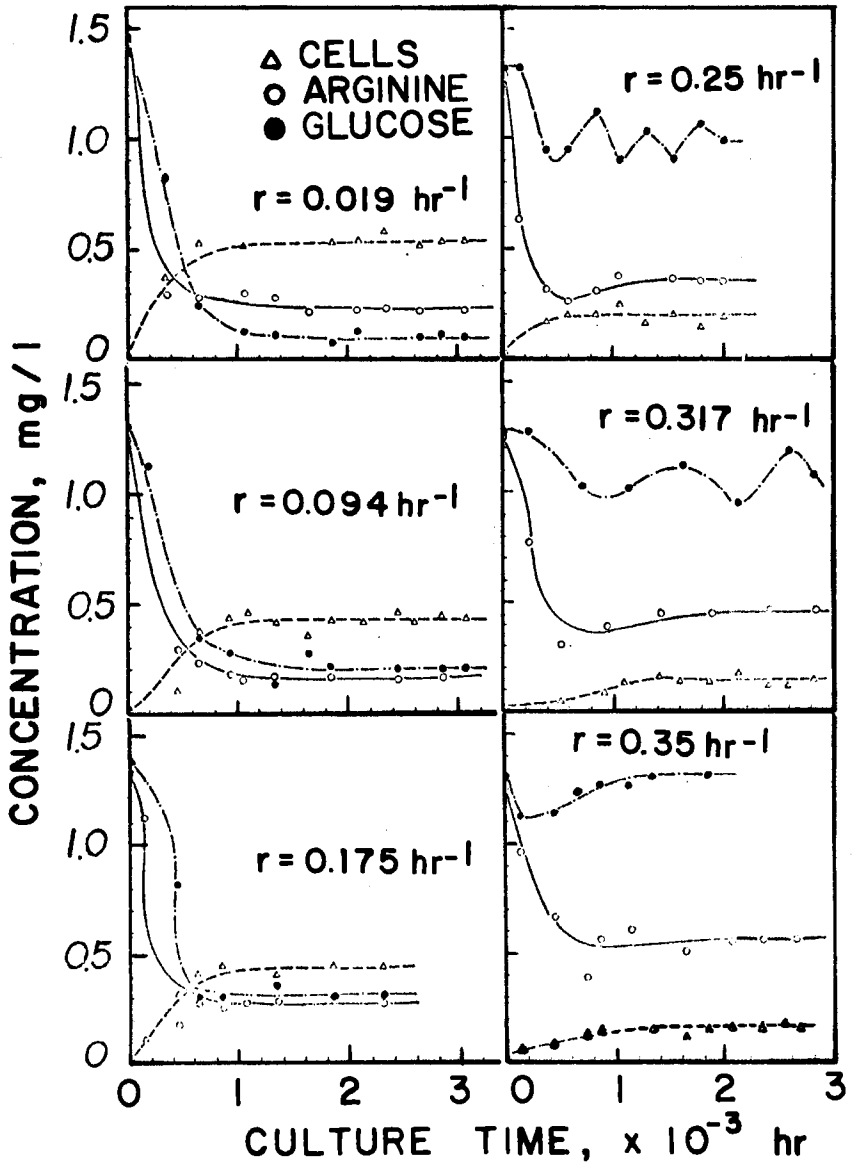

Figure 22. Time course of arginine-glucose limited continuous culture at various growth rates. The organisms were allowed to grow for 10 hours in batch culture in the reactor before beginning medium flow.

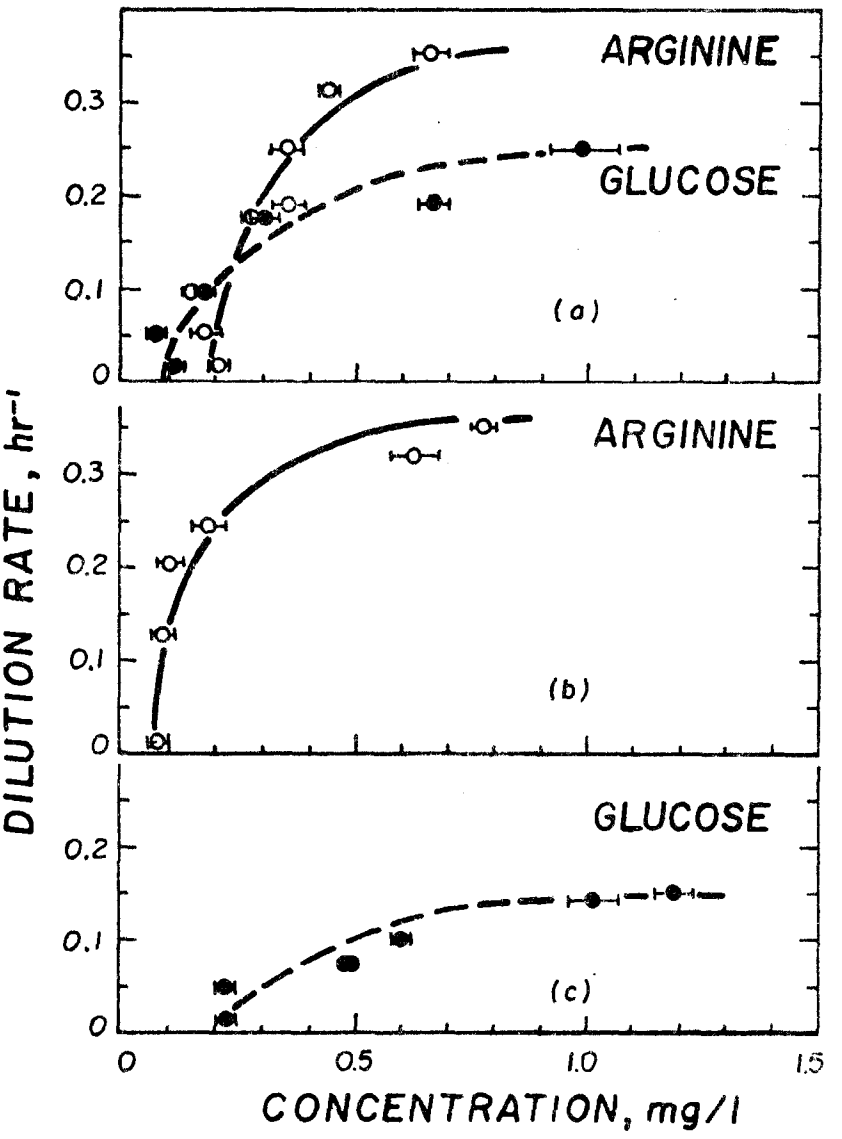

Figure 23. The relationship between growth rate and steady state arginine and glucose concentrations: (a) in arginine-glucose limited continuous culture; (b) in arginine limited continuous culture; (c) in glucose limited continuous culture.

observed in the case of arginine limited growth.

During dual substrate culture one of the substrate threshold values increased (arginine, from 0.07 to 0.17 mg/l) and the other decreased (glucose, from 0.2 to 0.08 mg/l) as compared with the threshold concentrations when each was the sole limiting substrate individually.

Figure 24 shows the response of pool material concentration with respect to dilution rate at steady state during both arginine and arginine-glucose limited growth. This figure reflects that pool material concentration is under the same type of regulation during arginine-glucose limited growth as during arginine limited growth alone. The relationship between pool glutamic acid concentration and dilution rate also follows the same pattern as that of arginine limited growth alone presented earlier. Figure 25 shows that pool glutamic acid was derived exclusively from arginine. This observation was revealed from the experiments where only arginine was labeled rather than both substrates were labeled. Since only arginine was labeled, the [^{14}C]glutamic acid isolated from the pool material was provided solely by arginine. The cell mass contributed by labeled arginine was estimated from the filter radioactivity with Equation (27); while, those portions of cell mass contributed by glucose were calculated from the glucose yield constant, obtained from the experiments where both substrates were labeled (0.217 g cell produced/g glucose consumed), and the amount of glucose used. The pool glutamic

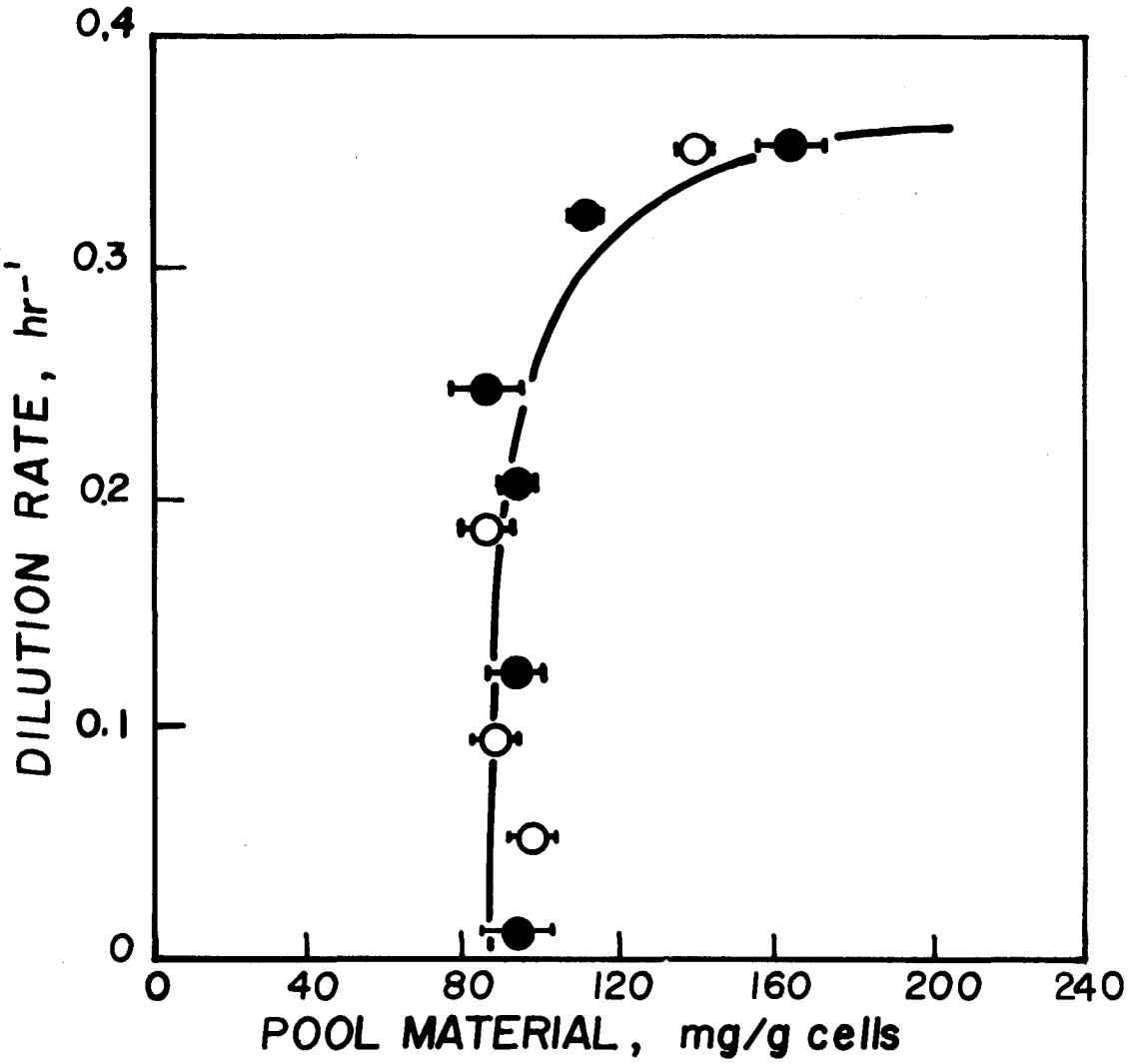

Figure 24. The relationship between the growth rate and the intracellular substrate concentration in arginine-glucose limited continuous culture (O). The data obtained from arginine limited growth are included in the figure for comparison (●).

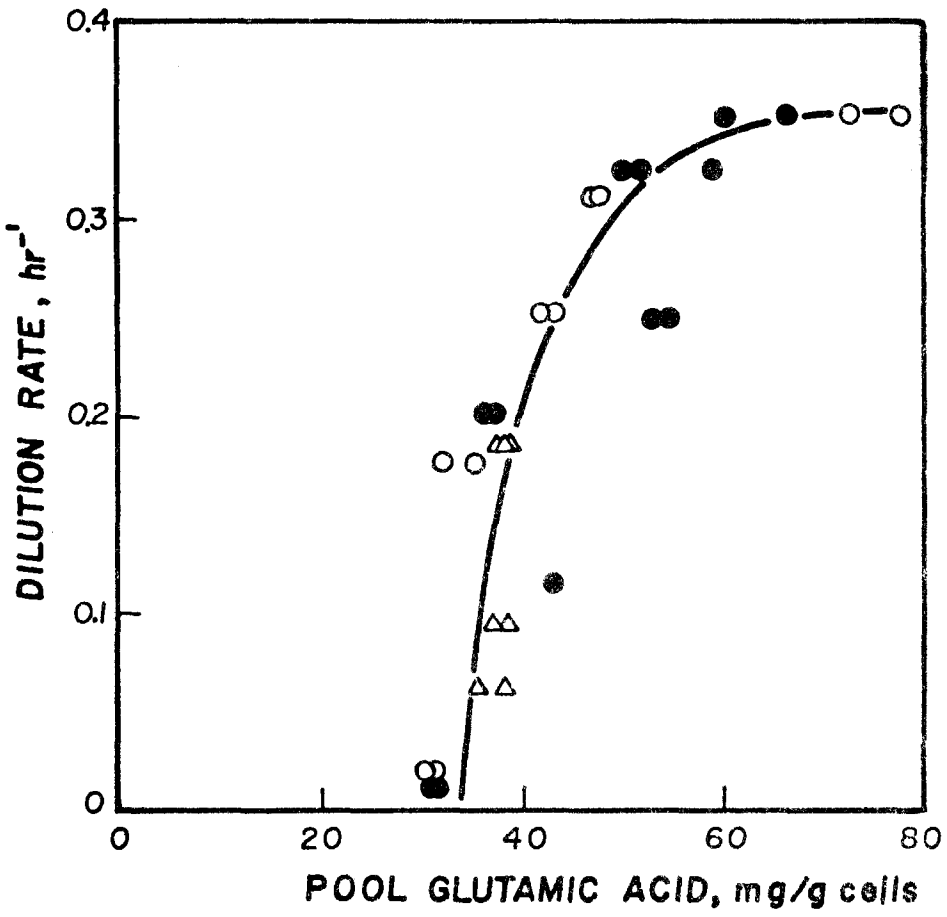

Figure 25. The relationship between growth rate and intracellular glutamic acid concentration in arginine-glucose limited continuous culture.
Symbols: ○ [^{14}C]glutamic acid derived from the experiment where both substrates were uniformly ^{14}C labeled; △ [^{14}C]glutamic acid derived from the experiment where only arginine was uniformly ^{14}C labeled; ● [^{14}C]glutamic acid derived from [U-^{14}C]arginine limited continuous culture, included here for comparison.

acid concentration (mg [^{14}C]glutamic acid/g cells, dry) with respect to dilution rate falls on the same curve where both substrates were labeled. The results can be seen in Figure 25.

The results of this study are summarized in Table 7 and detailed results can be found in Appendix C.

Continuous culture in arginine-glucose-glutamic acid limiting medium

Glutamic acid occupied a major portion of the pool material during arginine limited, glucose limited and arginine-glucose limited growth. How do the organisms metabolize intercellular glutamic acid, if it is one of the limiting substrates? This question was investigated with an arginine-glucose-glutamic acid limiting continuous culture at low growth rate with equal amounts of arginine, glucose and glutamic acid supplied to the fresh medium.

Figure 26 shows the results of the experiment. Intercellular arginine was metabolized efficiently at the beginning of the experiment while glutamic acid was consumed at a much slower rate. However, after the organisms started growing actively, they utilized glutamic acid instead of arginine. When the steady state was finally achieved after 200 hours, only a small amount of arginine was consumed. Apparently, the glutamic acid transport system is inducible. Intercellular glutamic acid can supply pool glutamic acid directly; therefore, when the glutamic acid transport system was induced the organisms harvested glutamic acid in preference to arginine from the

Table 7. Steady state intercellular and intracellular concentrations in arginine-glucose limited continuous culture.

	Intercellular		Intracellular	
Growth rate (hr^{-1})	Arginine (mg/l)	Glucose (mg/l)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.019	0.21 ± 0.02	0.11 ± 0.02	--	30.5[1]
0.057	0.18 ± 0.03	0.07 ± 0.01	109.4 ± 1.0	36.9
0.094	0.15 ± 0.01	0.19 ± 0.01	80.2 ± 6.2	37.0
0.175	0.28 ± 0.02	0.31 ± 0.03	--	33.2[1]
0.181	0.36 ± 0.04	0.67 ± 0.03	86.9 ± 5.7	38.4
0.250	0.36 ± 0.02	1.00 ± 0.08	--	43.5[1]
0.317	0.44 ± 0.02	--	--	60.5[1]
0.350	0.57 ± 0.04	--	130.0 ± 7.0	75.7

[1]Only arginine was ^{14}C-uniformly labeled in this experiment. The intracellular [^{14}C]glutamic acid was derived solely from [U-^{14}C]arginine.

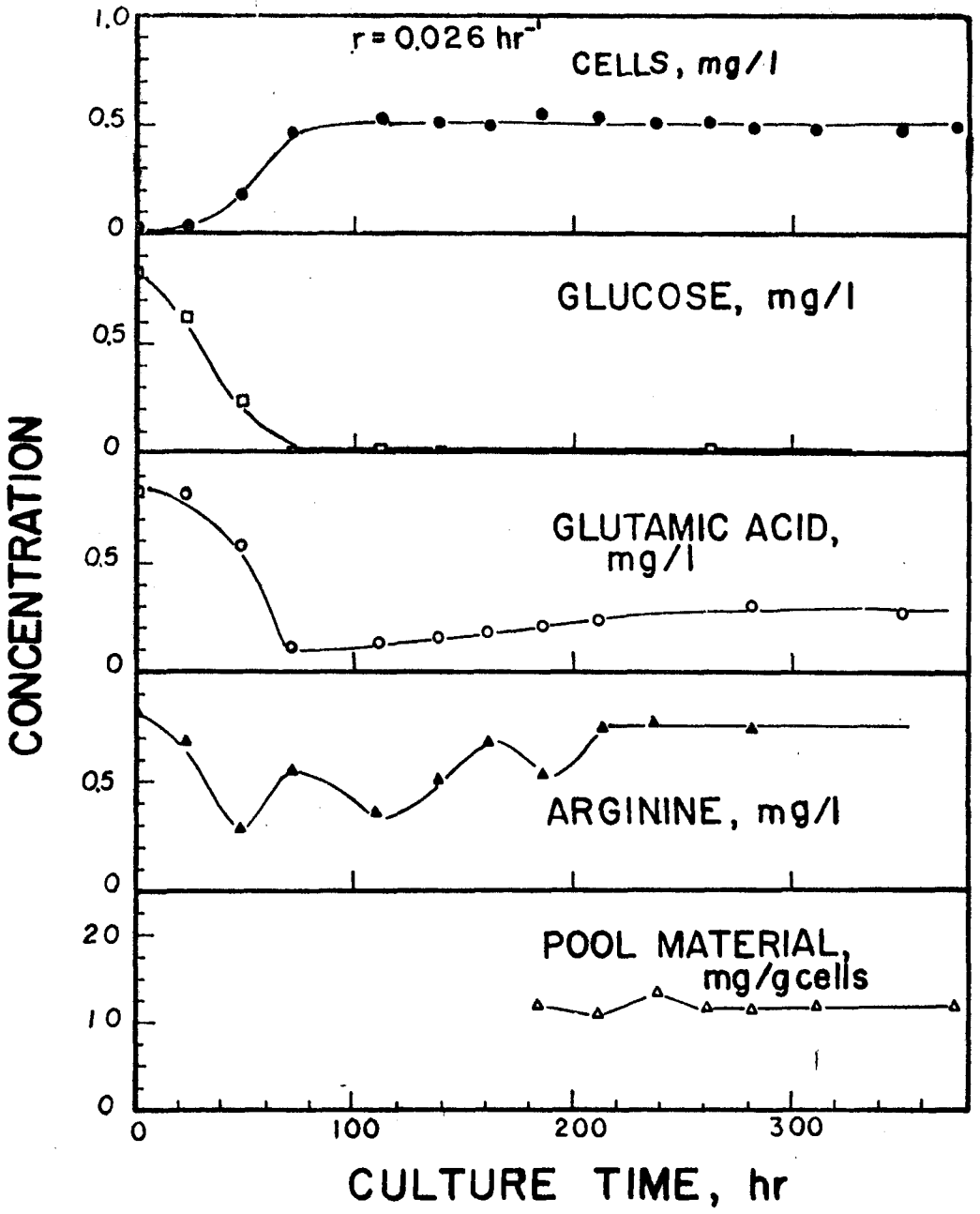

Figure 26. Time course of the arginine-glucose-glutamic acid limited continuous culture ($r=0.026\ hr^{-1}$). The organisms were allowed to grow for 10 hours in batch culture in the reactor before beginning medium flow.

medium. Little arginine was used at the steady state even though the system was carbon limited and the organisms had the capacity to use arginine.

Intercellular glucose concentration was almost exhausted in the reactor at steady state ($\leq$ 50 µg/l). This is an important finding; the threshold concentration declined from 0.2 to 0.08 mg glucose/l and then to a trace amount when the limiting substrate varied from glucose alone to glucose plus arginine and then to glucose plus arginine and glutamic acid.

At steady state, the organisms were able to maintain a steady state pool material concentration. The average value was 116.8 mg/g cells dry which was higher than the value obtained from arginine limited growth (90 mg/g cells) at that condition.

Figure 26 reveals that isolate 198 not only has the ability to metabolize multiple substrates simultaneously, but also to select the best available substrates from the environment to fit into its pool material requirements.

Continuous culture in glucose-mixed amino acids limiting medium

The growth of isolate 198 in multiple substrate was further studied in a glucose plus mixed amino acids limiting medium. This medium consisted of 1.7 mg/l of glucose and 1.0 mg/l of the mixed amino acids which contained equal amounts of all the 20 common L-amino acids. Since small amount of each individual L-amino acid was present

in the medium, only the intercellular glucose concentration was estimated in the study. Figure 27 shows the cell mass, the pool material and the intercellular glucose concentration from the experiment. As shown, the concentration of intercellular glucose at steady state was below the assay detection limits of 50 µg/l.

Pool material decay and organic by-products leakage during starvation of cells in batch culture

Experimental data suggest that whether in single substrate or in multiple substrate limited growth, there will be a considerable amount of pool material in the organism. An interesting question was raised: can isolate 198 maintain an appreciable amount of pool material during a long period of privation?

Cells, obtained from steady state culture of arginine-glucose-glutamic acid limited growth, were harvested by filtration with a 0.45 µm membrane filter. The cells were washed with 1% saline solution and then resuspended into the medium which contained no added carbon source. Cell mass, pool material and organic by-products leakage (estimated from the acidified filtrate radioactivity) from the cells were followed over a period of one week of starvation. Figure 28 shows the results of this experiment. The cell mass and the pool material decayed rapidly during the first five hours of starvation, but then remained fairly constant over the next seven days. The organic by-products excreted amounted to about 10% of the original cell

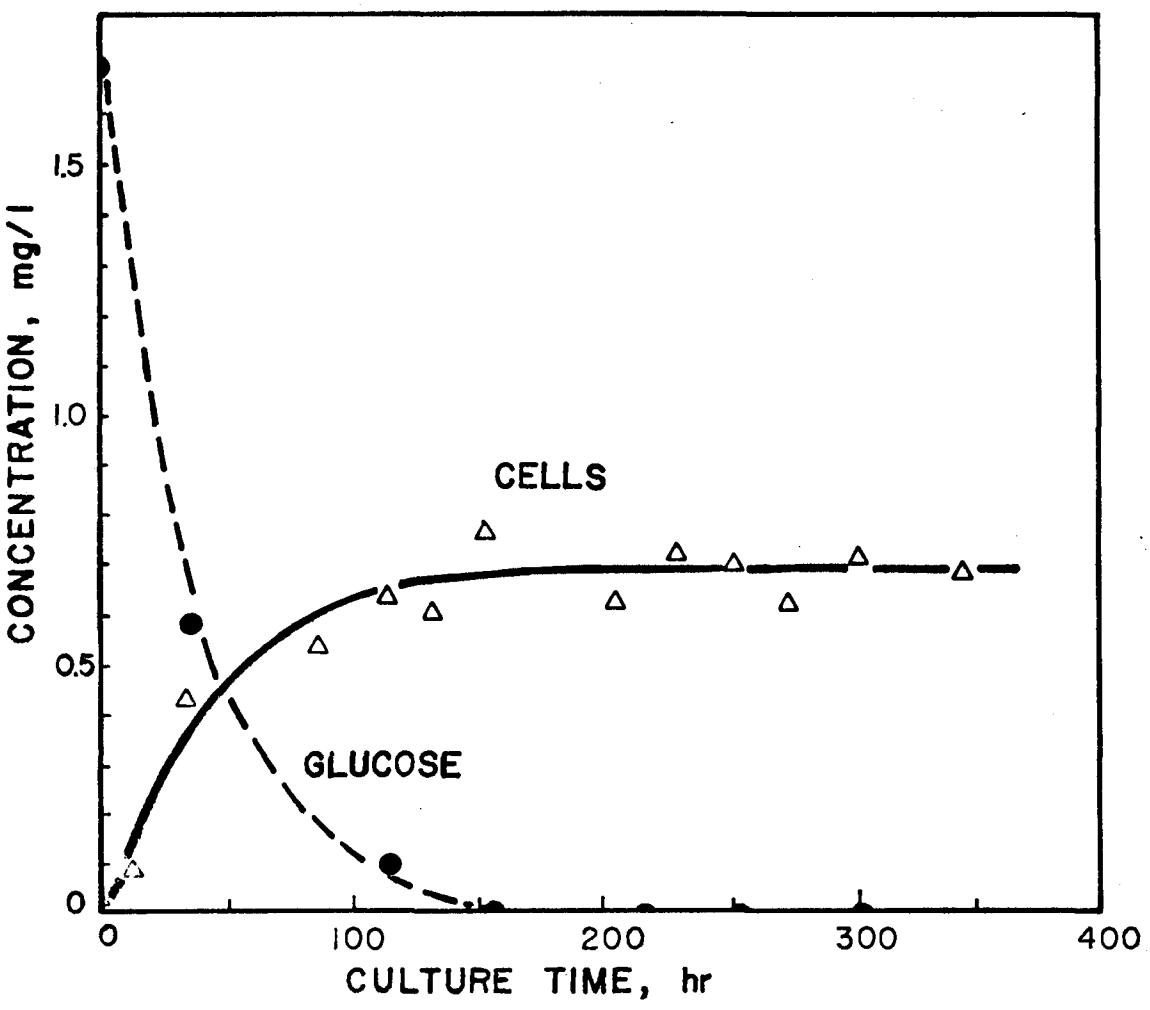

Figure 27. Time course of the glucose mixed amino acids limited continuous culture ($r=0.008\ hr^{-1}$). The organisms were allowed to grow for 10 hours in batch culture in the reactor before beginning medium flow.

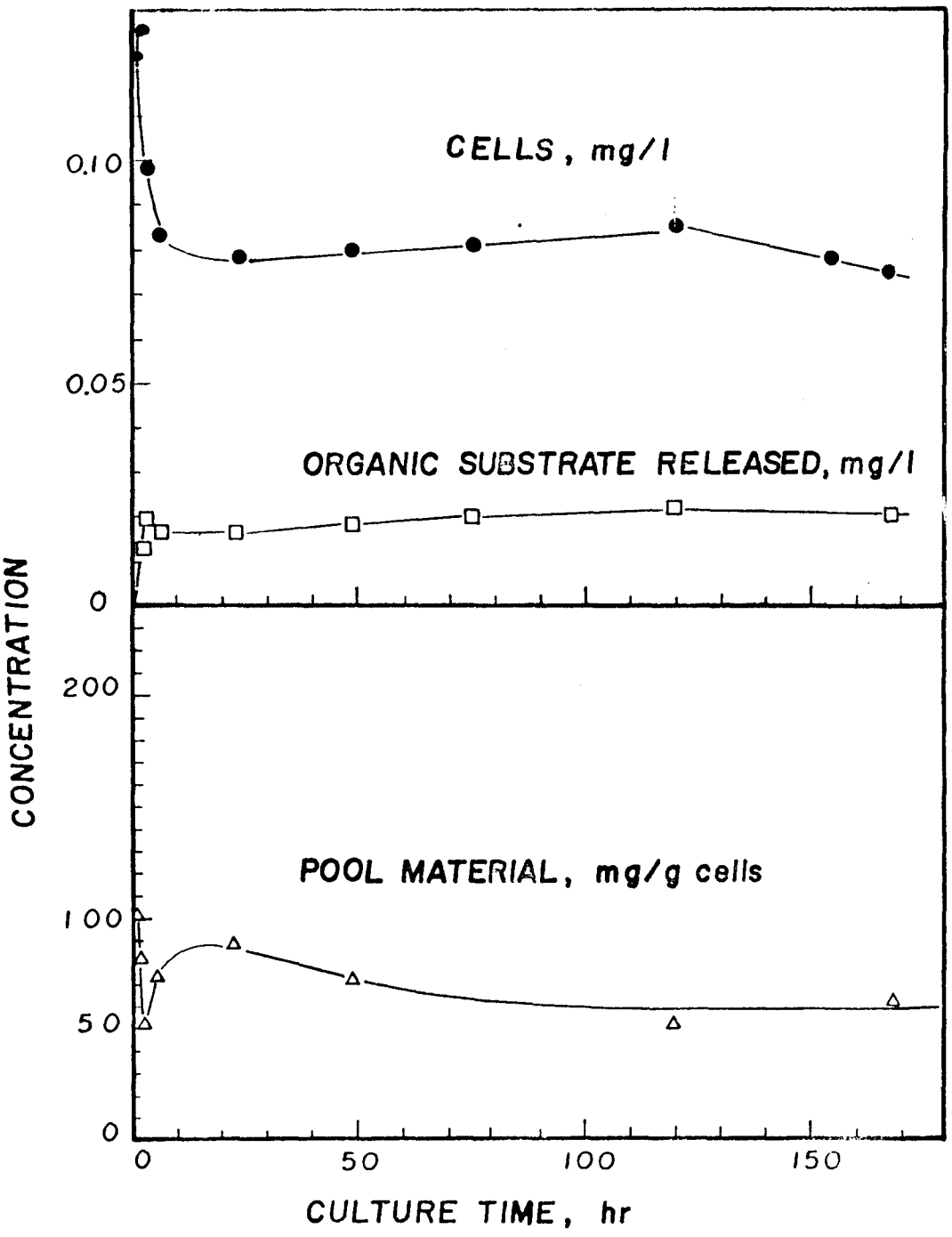

Figure 28. Variation of the intracellular substrate concentration, organic byproducts released, and cell mass during the starvation period of the organisms in the mineral medium.

mass during the period of rapid cell mass decay; thereafter, no further increase. Cell viability continued over the next three months without added carbon source.

DISCUSSION

With the enzymatic microanalyses developed here for glucose, arginine and glutamic acid, the classical works of Monod (1949) and Herbert et al. (1956), provide powerful techniques in kinetic studies of microbial growth in dilute multiple substrate solution. The microanalyses are simple; they require no desalting or concentration of the sample and they give sensitivity at the 50 µg/l level.

The glucose microanalysis is based on the hexokinase reaction which catalyzes the formation of glucose-6-phosphate from glucose and ATP. The glucose concentration was estimated from the amount of ATP removed from solution after glucose phosphorylation. The ATP concentration was measured from the response of light emissions of the luciferin-luciferase-ATP reaction with a scintillation counter. The lowest amount of ATP detected by this technique was about 1×10^{-10} g ATP/ml of the sample. Most of the glucose that normally contaminates commercial hexokinase was removed by treatment with glucose oxidase. The sensitivity of the glucose analysis was limited to the range of 50 µg glucose/l, possibly because of the trace amounts of glucose and the hexokinase reactive hexoses left after the treatment, as well as the trace amounts of phosphorylases associated with the hexokinase. By using more pure hexokinase preparation, glucose oxidase and luciferase, the sensitivity of the technique may improve to the range of 1 µg glucose/l.

The microanalyses of arginine and glutamic acid depend on the $^{14}CO_2$ released from the arginase-urease and the glutamic decarboxylase reactions. These techniques are very reproducible. The lowest detectable concentrations depend upon the specific activity of the amino acids added to the medium. In the arginine limiting growth studies, the concentration of arginine in the fresh medium was about 2.5 mg/l containing approximately 2000-3000 dpm/ml of [U-^{14}C]arginine. The lowest detectable arginine concentration in the medium by the enzyme based microanalysis was about 0.05 mg/l. The arginine microanalysis was developed for estimating the arginine concentration in a mixed amino acid saline solution. Therefore, to avoid the possibility of the commercial arginine decarboxylase carrying other amino acid decarboxylases, a combination of arginase and urease was employed for the arginine analysis.

Continuous culture is an excellent technique for microbial growth kinetic studies; however, this technique may lead to misleading conclusions if one disregards certain aspects of cell behavior as well as technical procedures. Since this study deals with extremely low organic concentrations, the sampling techniques, cell growth on the reactor wall, and background organics in the medium can cause significant errors in the estimation of intercellular substrate concentrations. Substrate depletion in the time interval between sample removal from the reactor and the metabolism arrest in the sample, as well as sample contamination resulting from unequilibrated medium,

were considered and analyzed theoretically. The significance of errors generated in the above sampling techniques can be seen by inspection of Equation (16) and (21). These errors were either completely avoided or reduced to a level which had no significant effect on the determination of the substrate concentrations.

The effect of reactor wall cell growth on the kinetic studies was analyzed and evaluated experimentally. Radioisotope substrate depletion experiments were used to evaluate the effect; however, the experimental data showed that trace amounts of cell growth on the reactor wall had no significant effect on the measured steady state substrate concentrations.

The background organic compounds in the medium were able to support a substantial cell population in batch culture and in continuous culture. Since isolate 198 has the ability to utilize various organic compounds simultaneously, these considerable amounts of background organics have a great effect on the amount of cell growth and the utilization kinetics of the added organics by the cells. A technique, which employed isolate 198 itself for scavenging the background organics was used. The treated medium supported only one hundredth of the cell population as compared to the untreated medium in continuous culture at very low growth rate. This treated medium was taken as acceptably carbon free. Double tap distilled prepared medium contains about 0.2 mg C/l in organic forms, and will support 10^5 cells/ml, while the described treatment yielded medium that would

support 10^3 cells/ml. It is unlikely that the remaining isolate 198 metabolically labile organics exceeded batch growth threshold concentrations by more than 0.002 mg C/l (0.2% of the added carbon content). These threshold concentrations are unknown, however, isolate 198 was observed to reduce dodecane to below 0.4 µg/l.

The tendency of isolate 198 to clump at high cell population prevented the application of electronic coulter counter and the standard microbiological plate count techniques for cell mass estimation. Direct weighing of the organisms was impossible because the routinely available sample volume was small and the cell mass (dry) was below 1.0 mg/l. Consequently, a ^{14}C-method based on the assumption that 50% of the dry cell material is carbon and all the cells in continuous culture are viable was employed for the cell mass estimation. This is an excellent method for cell mass determination at low population because of the high sensitivity of radioactivity measurements.

This study investigates the growth kinetics of the marine isolate under conditions where the organic substrate was both comprised of multiple compounds and was very dilute. The intercellular limiting substrate concentrations, and formation of intracellular pool substrates at various growth rates were examined in detail. The complexity of intracellular pool formation and utilization has been discussed by Subramanian et al. (1973), and Cowie and Walton (1956). Subramanian et al. (1973) observed that Neurospora rapidly used external or biosynthetic [^{14}C]arginine for protein synthesis without

mixing with the bulk of the large endogenous pool of [^{12}C]arginine. After that the [^{14}C]arginine was selectively excluded from use in protein synthesis. Over 95% of intracellular arginine was found in the arginine containing organelle (Weiss, 1973), while the rest of the arginine was distributed in the cytoplasm. These results suggest that the cellular organization of Neurospora prevents the catabolism of arginine by storing the arginine in the organelle. However Cowie and Walton (1956) reported that when Torulopsis utilis grew in [^{14}C] fructose medium, certain intracellular amino acids (aspartic acid, glutamic acid, alanine and serine) were rapidly formed. Based on radioactivity measurements, Cowie and Walton (1956) found that the rate of the pool [^{14}C]amino acid incorporation into protein was not affected by the presence of exogenous [^{12}C]amino acids, leading them to the conclusion that in Torulopsis utilis the intracellular non-protein amino acids were adsorbed to larger molecules and the adsorption sites were intimately connected with the processes of protein synthesis.

In our work, the relationships between growth rate and intracellular pool substrate concentration were entirely different in arginine limited and glucose limited growth (see Figures 14 and 19). In arginine limited growth the response of growth rate to intracellular pool substrate concentration followed a hyperbolic function with a positive pool substrate intercept (the positive intercept or the threshold concentration will be discussed later). However, the

relationship was rather complicated in glucose limited growth; the intracellular pool substrate concentration had maximal values at low growth rates and minimal values at intermediate growth rates. So, even with the same microorganism, the kinetics of intracellular pool substrate formation and utilization in different limiting substrate concentrations vary widely. When the organisms grew on the mixed substrate of arginine-glucose, the intracellular pool substrate concentration as well as the concentration of the dominant pool constituent, glutamic acid, followed the same relationships with respect to the growth rates as when arginine was the scle limiting substrate. Surprisingly, all the pool glutamic acid was derived exclusively from the intercellular arginine although large amounts of glucose were used by the organisms at the same time providing more than 70% of the rest of pool constituents. However, in glucose limited growth a large amount of pool glutamic acid was still observed in the pool substrates and the pool glutamic acid approached a maximal value of low growth rate (see Figure 19) indicating that the organism has the ability to synthesize glutamic acid from glucose but it may have difficulty synthesizing those essential pool constituents which are derived from arginine or its derivatives. Therefore, in glucose limited growth a larger amount of glutamic acid is required to drive the reactions, especially at low growth rates where the enzyme levels become very low. The difficulty of the organism to grow on glucose alone can be seen from the lag phase of fifty hours observed in batch culture (Table 4);

in an arginine medium the lag phase was only ten hours, showing that the organism needs more time for intracellular organization in order to grow in a glucose medium. Perhaps the organism needs to accumulate enough glutamic acid to drive those reactions. Since the enzymatic reactions in the organism are in equilibrium (i.e. steady state culture) and because the organism has to synthesize more glutamic acid, the amount of low molecular weight metabolic intermediates (the intracellular pool substrate) would be expected to be higher in glucose limited than in arginine limited growth. The results show that not only is the intracellular pool substrate concentration higher than that in arginine limited growth (see Figures 14 and 19) but also that its concentration has a direct response to the pool glutamic acid (see Figure 19), perhaps explaining why the intracellular pool substrate concentration as well as the pool glutamic acid concentration were entirely different in glucose limited and arginine limited growth. Furthermore, in all experiments, pool glutamic acid comprised more than 30% of the total pool substrate, and a clear response of growth rate with the pool glutamic acid concentration was found in arginine limited and arginine-glucose limited growth (see Figures 15 and 25). Obviously, pool glutamic acid is the chief essential pool constituent for isolate 198 growth.

The ability of isolate 198 to utilize arginine, glutamic acid and glucose simultaneously is noteworthy because of the sequential or "diauxic" growth often observed in batch culture (Monod, 1949; Stumm-

Zollinger and Harris, 1971; Standing et al., 1972). In the ocean, each species of dissolved organic compounds is present at very low concentrations (1 μg C/l or less). A microorganism utilizing the dissolved organic compounds sequentially would have difficulty surviving because the probability of collision between an organism and a particular substrate is very low. Therefore, it is not surprising to find that isolate 198 utilizes the multiple substrates at low concentrations simultaneously rather than sequentially. Standing et al. (1972) found that Escherichia coli, which utilized glucose and xylose or glucose and galactose sequentially in batch culture, consumed both substrates completely in continuous culture. This probably occurred because the small amount of glucose at steady state was not sufficient to cause the inhibition of either xylose or galactose utilization. In our work, however, the utilization of arginine and glucose simultaneously was not due to the fact that one of the substrates was reduced to a very low concentration. For example, at a growth rate of 0.175 hr^{-1} [see Figure 23(a)] the steady state concentrations of arginine and glucose were 0.25 mg/l and 0.3 mg/l respectively. Furthermore, isolate 198 reduced the steady state glucose concentration to a lower value as the number of limiting organic compounds increased in the medium. As can be seen from Figure 23(a,b), lower steady state glucose concentrations were observed in arginine-glucose limited growth as compared to glucose limited growth. Further reduction of steady glucose concentration was found in arginine-glucose-glutamic

acid limited growth (Figure 26) as well as in the glucose-mixed amino acid limited growth (Figure 27). In arginine-glucose-glutamic acid limited growth ($r = 0.026\ hr^{-1}$) the steady state glucose concentration was 0.05 mg/l as compared to 0.1 mg/l in arginine-glucose limited growth at the same growth rate. In glucose-mixed amino acid limited growth ($r = 0.008\ hr^{-1}$), the glucose concentration was below the sensitivity of the glucose analysis ($\leq$0.05 mg/l), but the glucose concentration was 0.08 mg/l at the same growth rate in arginine-glucose limited growth. However, as can be seen from Figure 23(a) the affinity for arginine in the presence of glucose was less than that when arginine was the sole limiting substrate. The higher affinity for glucose and the lower affinity for arginine in arginine-glucose limited growth can be further clarified in the following consideration. The utilization rates of glucose and arginine at steady state ($\tilde{f}_A$) can be calculated from the equation,

$$\tilde{f}_A = \frac{F(A_o - \tilde{A}_{out})}{V\tilde{X}} = \frac{r(A_o - \tilde{A}_{out})}{\tilde{X}} \qquad (32)$$

where $\tilde{f}_A$ is the utilization rate (g substrate/g cells x hr). The utilization rates of glucose and arginine, calculated from Equation (32), in single and dual substrate continuous culture are summarized in Table 8 and shown in Figure 29(a,b). Figure 29(a) clearly shows that the affinity for arginine was lower in the presence of glucose. For example, the utilization rate of arginine was 1.25 in the arginine

Table 8. Steady state utilization rates of glucose and arginine in various substrate limited continuous culture[1].

Arginine-glucose limited growth				Arginine limited growth		Glucose limited growth	
$\tilde{A}_{arg}$[2] (mg/l)	$\tilde{f}_{arg}$[3] (hr^{-1})	$\tilde{A}_{glu}$[2] (mg/l)	$\tilde{f}_{glu}$[3] (hr^{-1})	$\tilde{A}_{arg}$ (mg/l)	$\tilde{f}_{arg}$ (hr^{-1})	$\tilde{A}_{glu}$ (mg/l)	$\tilde{f}_{glu}$ (hr^{-1})
0.21	0.05	0.10	0.05	0.07	0.06	0.22	0.06
0.18	0.11	0.07	0.13	0.08	0.49	0.23	0.41
0.15	0.24	0.19	0.25	0.10	0.85	0.50	0.38
0.28	0.41	0.30	0.42	0.19	1.20	0.60	1.03
0.36	0.37	0.66	0.28	0.63	1.20	1.05	1.20
0.36	1.37	1.00	0.47	0.78	1.81	1.13	1.33
0.44	2.00	1.10	0.25				
0.56	1.42	1.17	0.00				

[1]In an arginine-glucose-glutamic acid limited continuous culture ($r=0.026\ hr^{-1}$), $\tilde{A}_{glu} \leq 0.05$ mg/l, $\tilde{f}_{glu}=0.04\ hr^{-1}$. In a glucose-mixed amino acids limited continuous culture ($r=0.008\ hr^{-1}$), $\tilde{A}_{glu}<0.05$ mg/l, $\tilde{f}_{glu}=0.02\ hr^{-1}$.

[2]Steady state concentration of arginine ($\tilde{A}_{arg}$) and glucose ($\tilde{A}_{glu}$).

[3]Steady state utilization rate (g substrate utilized/g cells/hr) of arginine ($\tilde{f}_{arg}$) and glucose ($\tilde{f}_{glu}$).

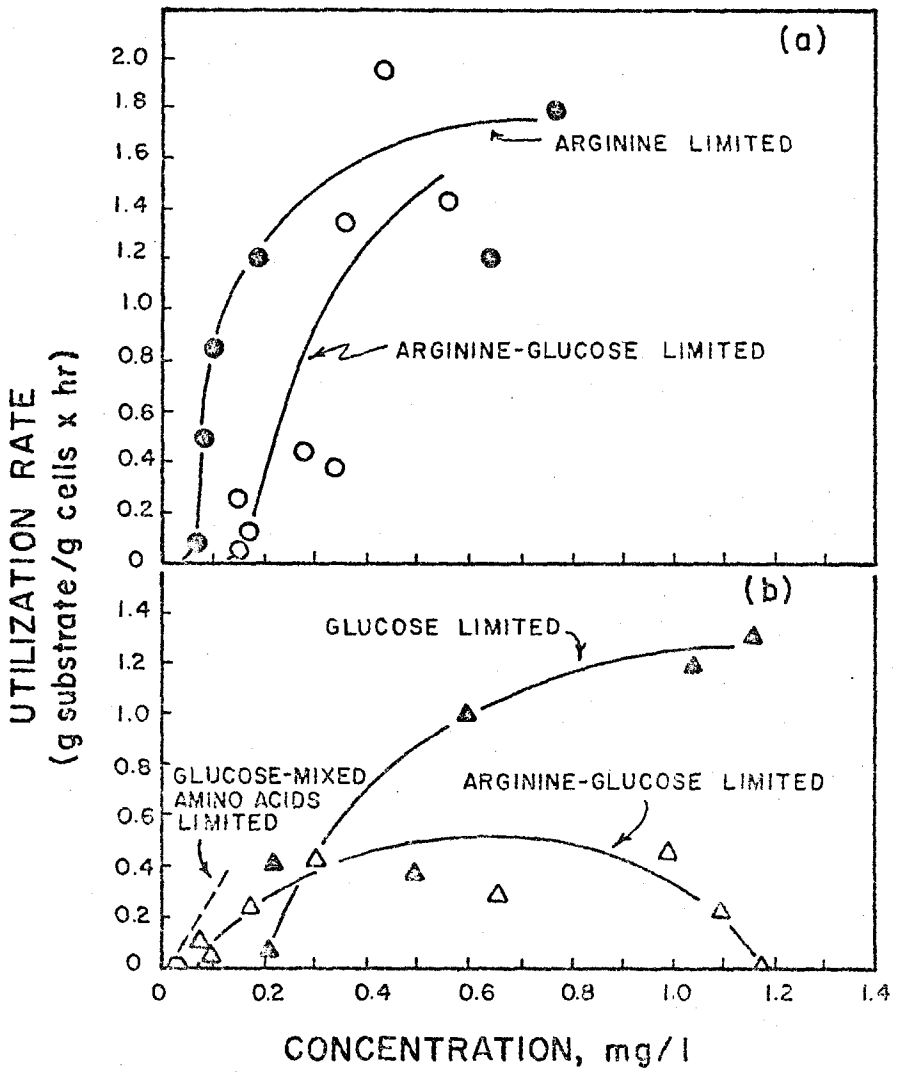

Figure 29. Effect of utilization rates of arginine and glucose in multiple substrate limited growth: (a) utilization rate of arginine in arginine limited and arginine-glucose limited growth; (b) utilization rate of glucose in glucose limited, arginine-glucose limited and glucose mixed amino acids limited growth.

limited growth at 0.2 mg arginine/l; however, in arginine-glucose limited growth at the same arginine concentration the utilization rate was 0.2 only. Figure 29(a) also shows that at high growth rates ($r>0.25\ hr^{-1}$), where no considerable amount of glucose was used, the utilization rates of arginine approached those obtained when arginine was the sole limiting substrate. Apparently, the arginine transport system was repressed by the presence of glucose in low arginine concentration. In high arginine concentration (high growth rate) the effect of glucose on the utilization rate of arginine disappeared although there was a high amount of glucose in the medium. Figure 29(b) shows that the organism developed a higher affinity for glucose in the presence of arginine at low glucose concentration (<0.2 mg/l). For example, at 0.15 mg glucose/l the utilization rate of glucose was 0.2 g glucose/g cells x hr in the presence of arginine; however, it was zero in glucose limited growth. Further increments of glucose affinity were found as the number of limiting organic substrates increased in the medium. The utilization rates of glucose in arginine-glucose-glutamic acid and in glucose-mixed amino acid limited growth were included in Figure 29(b) to show that increase. Figure 29(b) also indicates that the maximum utilization rate of glucose was much higher in glucose limited growth as compared with that in the arginine-glucose limited growth; the values were 1.3 and 0.45 g glucose/g cells x hr, respectively. The reason for reducing the maximum utilization rate of glucose is not known. Probably at high growth rates the

organism devoted all its ability for the arginine transport system. As can be seen from Figure 22, when the growth rate was greater than 0.25 hr^{-1}, the organism began losing its ability to utilize intercellular glucose and lost all its ability when the growth rate was raised to 0.35 hr^{-1}. The ability of isolate 198 to reduce glucose to an extremely low concentration in multiple substrate solution is very interesting and important. Perhaps in the ocean some marine heterotrophic microorganisms like isolate 198 are responsible for reducing several organic compounds to extremely low concentration while other species of microorganisms have higher affinity for other groups of organic compounds. Consequently, the concentration of the total dissolved organic compounds is very low in natural waters; much lower than one would anticipate from kinetic data derived from single limiting substrate systems.

In a multiple substrate medium, the organism apparently possesses the ability to select the best available intercellular substrate to fulfill its metabolic pool substrate requirements as reflected by its pool concentrations. This was revealed by the arginine-glucose-glutamic acid limited growth study (see Figure 26); glutamic acid is the dominant intracellular constituent. The most efficient way to provide this pool with glutamic acid is to harvest any intercellular glutamic acid present in the medium; another possible way is to harvest those compounds, like arginine, which can easily be transformed into glutamic acid. The glutamic acid transport system of

isolate 198 is apparently inducible. After induction of the system, the organism utilizes intercellular glutamic acid instead of arginine while the transport system for arginine is apparently repressed by the intracellular glutamic acid supplied by the medium. Thus, the organism appears to be able not only to utilize many substrates when all are present at low concentrations, but also to select the most suitable substrates from among them if those substrates appear in sufficient concentration to support moderate growth rates.

The threshold concentration of a limiting substrate is defined as that substrate concentration below which the organism are unable to grow (i.e. growth rate is zero). In studying the continuous culture of several marine bacteria with organic carbon enriched sea water, Jannasch (1967) observed that when the added organic compound was reduced to a certain level (0.5-100 mg/l) a washout of the organism occurred. The threshold concentrations of lactate, glycerol and glucose were between 0.5 to 100 mg/l, much higher than the values expected in the ocean. In our work, although the total amount of organic compounds added to the mineral medium was very low (2.5 mg/l), there was no washout at low growth rates. However, clear threshold concentrations were observed by extrapolating the growth rate vs. substrate curves [see Figure 23(a,b,c)] to zero growth rate. Threshold concentrations of nitrate and ammonium based on computation from transient state uptake kinetics have been reported for several phytoplankters in nitrogen limiting medium (Caperon and Meyer, 1972). Why threshold

of pool substrate besides that required to maintain the organism for survival is necessary as the driving force to support growth processes. The substrate concentration, A_f, which is required to maintain the critical amount of intracellular substrate concentration, $S_{p,f}$, for growth is the threshold concentration. When $A_{out}<A_f$ no growth would be expected because the flux of substrate can not satisfy the critical pool material requirement [i.e., $S_p<S_{p,f}$; see Figure 30(b)]. Under this critical condition (i.e., $A_{out}<A_f$), the organism may well reduce its metabolism as much as possible, thereby utilizing the substrate slowly and allowing it to survive for a much longer period of time. This explanation may account for not only the organic but also the inorganic threshold concentrations which have been observed in natural waters. For example, when nitrogen is the limiting substrate in sea water and its concentration drops below that needed to provide the minimum amount of pool nitrogen to sustain the growth of phytoplankton, growth ceases and the photosynthetic rate decreases to the level which is required only for endogeneous processes. Consequently, a threshold concentration of nitrogen concentration is established below which organisms exist in a viable but non-growing state.

Threshold concentrations of arginine and glucose, obtained from extrapolating, were observed not only when each was present as the sole limiting carbon source but also when they were present together in limiting concentrations. However, there was variation between the threshold concentrations found under each of the two conditions. The

results of the limiting substrate threshold concentrations (see Figure 23), summarized in Table 9, indicate that in the arginine-glucose limiting medium the threshold concentration of glucose decreases while that of arginine increases as compared to the values obtained when each was the sole limiting substrate. The reason for the variation of threshold concentrations probably is due to the fact that isolate 198 can utilize arginine and glucose simultaneously for growth, and because part of the pool of cell material is provided by arginine. The requirement of glucose by the same amount of cells will be less than that when glucose alone is limiting. Since flux of glucose into the cell depends on the ambient glucose concentration, a lower threshold concentration of glucose is expected in arginine-glucose limited as compared to glucose limited growth. As has been shown in Figure 29(a), the arginine transported system was repressed in the presence of glucose at low growth rates. Therefore, a higher arginine threshold concentration is expected in arginine-glucose than in arginine limited growth.

The variation of maximum growth rate between continuous culture and batch culture with the same amount of the limiting substrate is interesting and noteworthy. The organism grew much faster in continuous culture as compared to batch culture. In an arginine medium, a five-fold increment of maximum growth rate was observed while only a two-fold increment was detected in a glucose medium. A similar phenomenon has been observed with a marine yeast, R. rubra, in a

Table 9. Threshold concentrations of glucose and arginine in various limiting substrates continuous culture.

Condition	Glucose (mg/l)	Arginine (mg/l)
Arginine limiting	--	0.07
Glucose limiting	0.2	--
Arginine-glucose limiting	0.08	0.17
Glucose-mixed amino acids limiting[1]	<0.05	--[2]

[1]Results obtained from a continuous culture, growth rate 0.008 hr^{-1}.
[2]Not determined.

glucose medium (Law et al., 1971). Organic by-product inhibition of cell growth in bath is unlikely because the total organic concentration in the medium was low (2.5 mg/l). The chemical composition of the medium, the pH, temperature and the total organic compound added were the same in both types of cultures. The only difference was that one is a flow system and the other is a closed system (except for gases and heat exchange). Probably in continuous culture the organism has sufficient time to adjust its transport and metabolism capacities for maximum growth on the medium provided, while in batch culture, the substrate concentrations and the culture conditions vary rapidly and the organisms are never growing at their maximal capacities because they have insufficient time to adjust to the changes.

NOMENCLATURE

A_o	Substrate in fresh medium supply, g A/liter.
A_{out}	Average intercellular substrate concentration, g A/liter.
$\tilde{A}_{out}$	Steady state intercellular substrate concentration, g A/liter.
A_f	Threshold substrate concentration, g A/liter.
C_A	Carbon content of the substrate, percent.
$(dpm)_{CO_2}$	Disintegrations per minute of $^{14}CO_2$ collected per ml of sample added.
$(dpm)_p$	Disintegrations per minute of ^{14}C-pool extract per ml of culture.
$(dpm)_c$	Disintegrations per minute of ^{14}C-content of cell per ml of culture.
$(dpm)_{A_o}$	Disintegrations per minute of ^{14}C-substrate per ml of fresh medium.
E	Error in A_{out}, percent.
F	Fresh medium flow rate, liter/hr.
f_A	Flux of the limiting substrate, g A/g cells x hr.
$\tilde{f}_A$	Utilization rate of the limiting substrate at steady state, g A/g cells x hr.
$K_{0.5}$	Concentration of A_{out} when growth rate is half maximal, g A/liter.
M_p	A factor accounts for the assumption that 50% of the pool material (dry) is carbon.
M_c	A factor accounts for the assumption that 50% of the cell material (dry) is carbon.
N	The ratio between atom carbon per molecule and atom carbon cleaved per molecule.

Q	Reactor sample withdrawal rate, ml/sec.
r	Reactor dilution rate, hr^{-1}.
t	Time.
$t_{1/2}$	Time required for a process to reduce by one half.
μ	Growth rate, hr^{-1}.
μ_{max}	Maximum growth rate, hr^{-1}.
V	Volume of reactor, liter.
V_w	Volume occupied by cells growing on the reactor wall, liter.
W	Reactor sampling dilution rate, sec^{-1}.
X	Dry cell mass, g/liter.
$\tilde{X}$	Steady state dry cell mass, g/liter.
Y_{aA}	Apparent yield of cells produced from substrate utilized, g cells/g substrate.

Appendix A

Table A-1. Intercellular and intracellular concentrations during the course of an arginine limited continuous culture, growth rate 0.01 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Urea (g/g cells)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.05	1.97	--	--	--
25.0	0.33	--	--	--	--
38.0	0.32	--	--	--	--
107.0	0.33	0.12	--	--	--
134.0	0.31	0.11	--	--	--
178.0	0.34	0.08	--	84	--
231.0	0.28	0.08	--	106	--
278.0	0.35	0.06	2.2	108	--
327.0	0.35	0.07	2.0	104	--
375.0	0.38	--	--	--	--
448.0	0.34	0.06	2.0	--	--
495.0	0.34	0.09	2.0	--	--
544.0	0.32	--	--	84	--
617.0	0.30	--	--	108	31.5, 32.5

Table A-2. Intercellular and intracellular concentrations during the course of an arginine limited continuous culture, growth rate 0.123 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Urea (g/g cells)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.05	2.59	--	--	--
24.0	0.36	--	--	--	--
43.0	0.61	0.08	--	--	--
92.0	0.75	0.12	--	93	--
159.0	0.58	0.07	--	102	--
207.0	0.67	--	--	97	--
256.0	0.53	--	--	99	--
351.0	0.62	0.08	--	--	--
399.0	0.66	--	--	--	--
495.0	0.75	--	0.8	--	--
548.0	0.65	0.10	--	85	--
592.0	0.66	--	0.8	--	--
714.0	0.60	0.07	0.9	99	--
737.0	0.61	--	0.9	86	43.7

Table A-3. Intercellular and intracellular concentrations during the course of an arginine limited continuous culture, growth rate 0.204 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Urea (g/g cells)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.05	2.51	--	--	--
43.0	0.47	--	--	--	--
71.0	0.54	--	--	--	--
96.0	0.59	--	--	--	--
114.0	0.52	--	--	--	--
239.0	0.63	--	--	--	--
408.0	0.59	--	0.8	--	--
451.0	0.60	0.1 ± 0.02[1]	0.9	93.4 ± 3.5[1]	36.2, 37

[1] Results from three analyses.

Table A-4. Intercellular and intracellular concentrations during the course of an arginine limited continuous culture, growth rate 0.246 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Urea (g/g cells)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.05	2.53	--	--	--
15.0	0.39	--	--	--	--
63.0	0.56	--	--	--	--
135.0	0.48	--	--	--	--
182.0	0.49	--	--	--	--
271.0	0.48	0.185 ± 0.03[1]	1.10 ± 0.02[1]	86 ± 10[1]	49.7, 48.4

[1] Results from three analyses.

Table A-5. Intercellular and intracellular concentrations during the course of an arginine limited continuous culture, growth rate 0.32 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Urea (g/g cells)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.02	2.51	--	--	--
22.0	0.04	--	--	--	--
43.0	0.08	--	--	--	--
70.0	0.39	--	--	--	--
102.0	0.44	--	--	--	--
114.0	0.47	--	--	--	--
138.0	0.61	--	--	--	--
180.0	0.49	--	--	--	--
259.0	0.58	--	--	--	--
357.0	0.47	--	--	--	--
377.0	0.49	--	--	--	--
448.0	0.49	0.63 ± 0.05[1]	0.61, 0.66	110 ± 2.0[1]	54 ± 5[1]

[1] Results from three analyses.

Table A-6. Intercellular and intracellular concentrations during the course of an arginine limited continuous culture, growth rate 0.35 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Urea (g/g cells)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.08	2.59	--	--	--
29.0	0.34	--	--	--	--
50.0	0.25	--	--	--	--
76.0	0.30	0.77	--	--	--
100.0	0.37	0.78	--	--	--
119.0	0.39	0.83	--	--	--
143.0	0.35	0.79	--	--	--
171.0	0.29	0.78	--	--	--
215.0	0.31	0.77	1.0	--	--
247.0	0.37	0.78	0.97	163 ± 9[1]	67, 60

[1] Results from three analyses.

Appendix B

Table B-1. Intercellular and intracellular concentrations during the course of a glucose limited continuous culture, growth rate 0.016 hr^{-1}.

		Intercellular			Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Glucose (mg/l)	Byproducts (g/g cells)	CO_2	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.02	2.49	--	--	--	--
90.5	0.44	0.43	--	--	--	--
110.5	0.49	--	--	--	--	--
134.5	0.56	--	--	--	--	--
158.0	0.61	0.22	--	--	--	--
182.0	0.65	--	--	--	--	--
206.0	0.62	0.22	0.90	2.5	--	--
230.0	0.58	0.24	--	--	--	--
254.0	0.64	0.24	0.94	2.4	--	--
283.0	0.56	0.20	--	--	--	--
302.0	0.59	0.22	0.94	2.8	141 ± 10[1]	55.8, 51.4

[1] Results from three analyses.

Table B-2. Intercellular and intracellular concentrations during the course of a glucose limited continuous culture, growth rate 0.073 hr^{-1}.

		Intercellular			Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Glucose (mg/l)	Byproducts (g/g cells)	CO_2	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.02	2.53	--	--	--	--
15.0	0.05	2.27	--	--	--	--
40.5	0.09	--	--	--	--	--
64.5	0.22	1.31	--	--	--	--
83.5	0.43	0.77	--	--	--	--
108.5	0.44	0.50	0.95	3.9	--	--
137.0	0.41	--	--	--	--	--
157.0	0.42	0.49	1.09	4.1	71.9 ± 13[1]	35.9, 38

[1] Results from three analyses.

Table B-3. Intercellular and intracellular concentrations during the course of a glucose limited continuous culture, growth rate 0.148 hr^{-1}.

		Intercellular			Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Glucose (mg/l)	Byproducts (g/g cells)	CO_2	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.02	2.67	--	--	--	--
50.0	0.06	2.15	--	--	--	--
71.5	0.10	--	--	--	--	--
96.0	0.18	1.24	--	--	--	--
118.0	0.24	--	--	--	--	--
143.5	0.25	1.23	0.8	5.0	--	--
185.0	--	0.95	--	--	--	--
215.5	0.20	1.04	1.4	7.7	--	--
227.0	0.20	1.05	1.3	7.9	--	--
250.0	0.21	--	--	--	104.8 ± 13[1]	43.5, 41.5

[1] Results from three analyses.

Table B-4. Intercellular and intracellular concentrations during the course of a glucose limiting continuous culture, growth rates 0.052, 0.10, and 0.15 hr^{-1}.

		Intercellular			Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Glucose (mg/l)	Byproducts (g/g cells)	CO_2	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
			Growth rate 0.10 hr^{-1}			
0.0	0.02	2.55	--	--	--	--
60.0	0.19	1.12	--	--	--	--
108.5	0.25	0.60	--	--	--	--
132.0	0.23	--	--	--	--	--
156.0	0.23	0.59	1.51	9.8	--	--
180.0	0.19	--	--	--	--	--
203.0	0.18	0.60	1.84	12.5	--	--
229.0[1]	0.19	--	--	--	85.2 ± 11[2]	24.3, 26.7
			Growth rate 0.052 hr^{-1}			
277.5	0.15	1.40	--	--	--	--
325.5	0.24	--	--	--	--	--
349.0	0.26	0.25	--	--	--	--
374.0	0.29	0.21	1.0	9.8	--	--
421.0	0.29	0.22	1.1	9.6	--	--
449.0[1]	0.30	--	--	--	116, 124.7	24, 23.9

Table B-4 (continued)

Culture time (hr)	Dry cell mass (mg/l)	Intercellular			Intracellular	
		Glucose (mg/l)	Byproducts (g/g cells)	CO_2	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
472.0	0.12	1.30	--	--	--	--
518.5	0.17	--	--	--	--	--
589.0	0.20	--	--	--	--	--
615.0	0.15	--	--	--	--	--
622.0	0.13	1.17	1.0	11	--	--
689.5	0.17	1.22	--	--	--	--
715.0	0.16	1.13	1.2	11.1	--	--

1 Removed 100 ml of culture from the reactor (200 ml) for pool extraction. The void volume was then filled with fresh medium for the following run.

2 Results from three analyses.

Appendix C

Table C-1. Intercellular and intracellular concentrations during the course of an arginine-glucose[1] limited continuous culture, growth rate 0.019 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Soluble material[2] (mg/g cells)	Glutamic acid[3] (mg/g cells)
0.0	0.02	1.50	1.40	--	--
37.0	0.39	0.29	0.83	--	--
67.0	0.53	0.29	0.24	--	--
109.0	0.52	0.32	0.12	--	--
133.0	0.50	0.28	0.11	--	--
189.0	0.53	--	0.07	--	--
210.0	0.54	0.22	0.12	54	--
237.0	0.59	0.23	0.10	43	--
263.0	0.52	0.22	0.11	57	--
284.0	0.55	--	0.09	61	--
309.0	0.54	0.23	0.14	54	--
326.0	0.54	0.22	0.07	--	--
370.0	0.51	0.21	--	--	--
448.0	0.53	--	--	--	--
496.0	0.53	--	0.11	--	--
523.0	0.46	0.17	0.13	53	30, 31

1 [U-^{14}C]arginine-[^{12}C]glucose experiment.

2 Soluble pool material derived from [U-^{14}C]arginine. Extract radioactivity is calculated as mg total TCA extract per g cells (dry weight).

3 Pool glutamic acid derived from [U-^{14}C]arginine.

Table C-2. Intercellular and intracellular concentrations during the course of an arginine-glucose[1] limited continuous culture, growth rate 0.057 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.02	1.24	1.31	--	--
48.0	0.60	--	--	--	--
71.5	0.42	0.18	0.08	109	--
95.5	0.58	0.28	0.05	--	--
122.0	0.39	0.19	0.10	125	--
143.5	0.52	0.13	0.07	109	--
167.0	0.57	0.21	--	--	--
195.0	0.58	--	0.07	101	--
215.0	0.52	0.19	0.06	--	--
226.0	0.54	0.18	0.07	103	36, 38

[1] [U-^{14}C]arginine-[U-^{14}C]glucose experiment.

Table C-3. Intercellular and intracellular concentrations during the course of an arginine-glucose[1] limited continuous culture, growth rate 0.094 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.02	1.24	1.31	--	--
45.5	0.11	0.30	1.23	--	--
68.5	0.39	0.23	0.35	75	--
92.5	0.44	0.19	0.27	83	--
110.0	0.46	0.15	0.27	70	--
139.5	0.41	0.18	0.13	83	--
165.5	0.36	0.16	0.26	102	--
188.0	0.43	0.15	0.22	--	--
212.5	0.44	0.16	--	85	--
247.0	0.46	0.14	0.19	--	--
260.0	0.43	0.14	0.18	86	--
283.0	0.45	0.16	0.20	--	--
308.5	0.43	--	0.19	--	37.3, 36.7

[1] [U-^{14}C]arginine-[U-^{14}C]glucose experiment.

Table C-4. Intercellular and intracellular concentrations during the course of an arginine-glucose[1] limited continuous culture, growth rate 0.175 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Soluble material[2] (mg/g cells)	Glutamic acid[3] (mg/g cells)
0.0	0.05	1.35	1.38	--	--
13.0	0.14	1.10	0.97	--	--
44.0	0.36	0.18	0.83	--	--
61.0	0.41	0.27	0.31	--	--
87.0	0.46	0.25	0.30	47	--
134.0	0.41	0.29	0.36	58	--
189.0	0.46	--	0.29	54	--
230.0	0.46	0.28	0.30	54	35, 32

1 [U-^{14}C]arginine-[^{12}C]glucose experiment.

2 Soluble pool material derived from [U-^{14}C]arginine. Extract radioactivity is calculated as mg total TCA extract per g cells (dry weight).

3 Pool glutamic acid derived from [U-^{14}C]arginine.

Table C-5. Intercellular and intracellular concentrations during the course of an arginine-glucose[1] limited continuous culture, growth rate 0.181 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Soluble material (mg/g cells)	Glutamic acid (mg/g cells)
0.0	0.02	1.00	1.17	--	--
36.0	0.04	0.06	--	--	--
60.0	0.21	--	0.98	--	--
84.0	0.29	0.24	--	--	--
108.0	0.23	--	--	--	--
160.0	0.31	--	0.70	84	--
184.0	0.35	0.38	--	91	--
205.0	0.34	--	0.66	82	--
227.0	0.32	0.38	--	83	--
253.0	0.34	0.32	0.65	95	40, 38, 39

[1] [U-^{14}C]arginine-[U-^{14}C]glucose experiment.

Table C-6. Intercellular and intracellular concentrations during the course of an arginine-glucose[1] limited continuous culture, growth rate 0.25 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Soluble material[2] (mg/g cells)	Glutamic acid[3] (mg/g cells)
0.0	0.02	1.28	1.32	--	--
40.0	0.18	0.33	0.94	--	--
61.0	0.21	0.26	0.94	--	--
84.0	0.23	0.33	1.13	--	--
107.0	0.25	0.39	0.99	68	--
132.0	0.17	--	1.04	85	--
155.5	0.20	0.37	0.93	92	--
179.0	0.15	0.35	1.07	--	--
202.0	0.19	0.35	1.00	92	43, 44

1 [U-^{14}C]arginine-[^{12}C]glucose experiment.

2 Soluble pool material derived from [U-^{14}C]arginine. Extract radioactivity is calculated as mg total TCA per g cells (dry weight).

3 Pool glutamic acid derived from [U-^{14}C]arginine.

Table C-7. Intercellular and intracellular concentrations during the course of an arginine-glucose[1] limited continuous culture, growth rate 0.317 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Soluble material[2] (mg/g cells)	Glutamic acid[3] (mg/g cells)
0.0	0.01	1.25	1.27	--	--
26.0	0.01	0.77	1.26	--	--
52.0	0.04	0.30	--	--	--
74.0	0.10	--	1.03	--	--
96.0	0.08	0.39	--	--	--
118.0	0.12	--	1.01	--	--
142.0	0.14	0.44	--	--	--
166.0	0.13	--	1.12	--	--
190.0	0.11	0.41	--	--	--
215.0	0.16	--	0.95	--	--
241.0	0.10	0.45	--	--	--
261.0	0.11	--	1.19	--	--
285.0	0.13	0.46	1.08	105, 110	47.5, 46.5

1 [U-^{14}C]arginine-[^{12}C]glucose experiment.

2 Soluble pool material derived from [U-^{14}C]arginine. Extract radioactivity is calculated as mg total TCA extract per g cells (dry weight).

3 Pool glutamic acid derived from [U-^{14}C]arginine.

Table C-8. Intercellular and intracellular concentrations during the course of an arginine-glucose[1] limited continuous culture, growth rate 0.35 hr^{-1}.

		Intercellular		Intracellular	
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Soluble material[2] (mg/g cells)	Glutamic acid[3] (mg/g cells)
0.0	0.02	1.25	1.30	--	--
17.0	0.07	0.96	1.12	--	--
42.0	0.09	0.67	1.13	--	--
74.0	0.13	0.38	--	--	--
89.0	0.16	0.56	1.24	--	--
112.0	0.15	0.60	1.26	--	--
137.0	0.16	--	1.30	--	--
162.0	0.14	0.50	--	--	--
184.0	0.15	--	1.30	--	--
207.0	0.18	0.55	--	--	--
231.0	0.16	0.56	--	--	--
254.0	0.18	0.63	--	--	--
266.0	0.17	0.56	--	130 $\pm$ 7	78, 73

1 [U-^{14}C]arginine-[^{12}C]glucose experiment.

2 Soluble pool material derived from [U-^{14}C]arginine. Extract radioactivity is calculated as mg total TCA per g cells (dry weight).

3 Pool glutamic acid derived from [U-^{14}C]arginine.

Appendix D

Table D. Intercellular and intracellular concentrations during the course of an arginine-glucose-glutamic acid limited continuous culture, growth rate 0.026 hr^{-1}.

		Intercellular			Intracellular
Culture time (hr)	Dry cell mass (mg/l)	Arginine (mg/l)	Glucose (mg/l)	Glutamic acid (mg/l)	Soluble material (mg/g cells)
0.0	0.02	0.81	0.82	0.82	--
24.0	0.04	0.70	0.63	0.82	--
48.0	0.20	0.29	0.23	0.58	--
70.5	0.44	0.55	≤0.05	0.11	--
114.5	0.53	0.36	0.05	0.15	--
139.0	0.53	0.52	≤0.05	0.16	--
163.0	0.50	0.70	--	0.20	--
186.0	0.56	0.54	--	0.20	119
211.0	0.54	0.73	--	0.23	110
238.0	0.52	0.75	--	--	132
263.0	0.49	0.73	0.05	0.31	116
331.0	0.48	--	--	0.29	113
356.5	0.48	--	--	0.27	120
379.0	0.50	--	--	--	117

Appendix E

Table E. Intercellular and intracellular concentrations during the course of a glucose-mixed amino acids limited continuous culture, growth rate rate 0.008 hr^{-1}.

		Intercellular	Intracellular
Culture time (hr)	Dry cell mass (mg/l)	Glucose (mg/l)	Soluble material (mg/g cells)
0.0	0.02	1.72	--
12.0	0.09	--	--
36.0	0.43	0.59	--
89.0	0.54	--	--
107.5	0.64	0.10	--
131.0	0.62	--	--
155.0	0.78	<0.05	--
208.0	0.62	<0.05	--
230.0	0.73	--	166.2
253.0	0.71	--	169.8
277.0	0.62	--	--
301.0	0.72	<0.05	170.0
349.0	0.68	--	192.0

Appendix F

A kinetic model for microbial growth

The simplified model is outlined in Figure 31. The derivation, based on the rates of formation of intracellular soluble material and their utilization for growth, includes the following formulations and assumptions.

A. f_1, the net flux, transport less substrate leakage, of an intercellular substrate into the pool.

Some of the intercellular substrates transported into the cytoplasm are immediately incorporated into macromolecules; others are transformed into intermediates which are then used for macromolecule biosynthesis. A substrate or intermediate having a long resident time accumulates in the soluble pool material, its concentration regulating the flux of additional substrate into the pool. Therefore, f_1 is assumed directly proportional to the intracellular soluble material concentration at a particular growth rate.

$$f_1 = C_1 \mu S_p X V$$

where,

μ = growth rate, hr^{-1}

S_p = intracellular soluble material concentration, g/g cells dry

X = dry cell mass, g/l

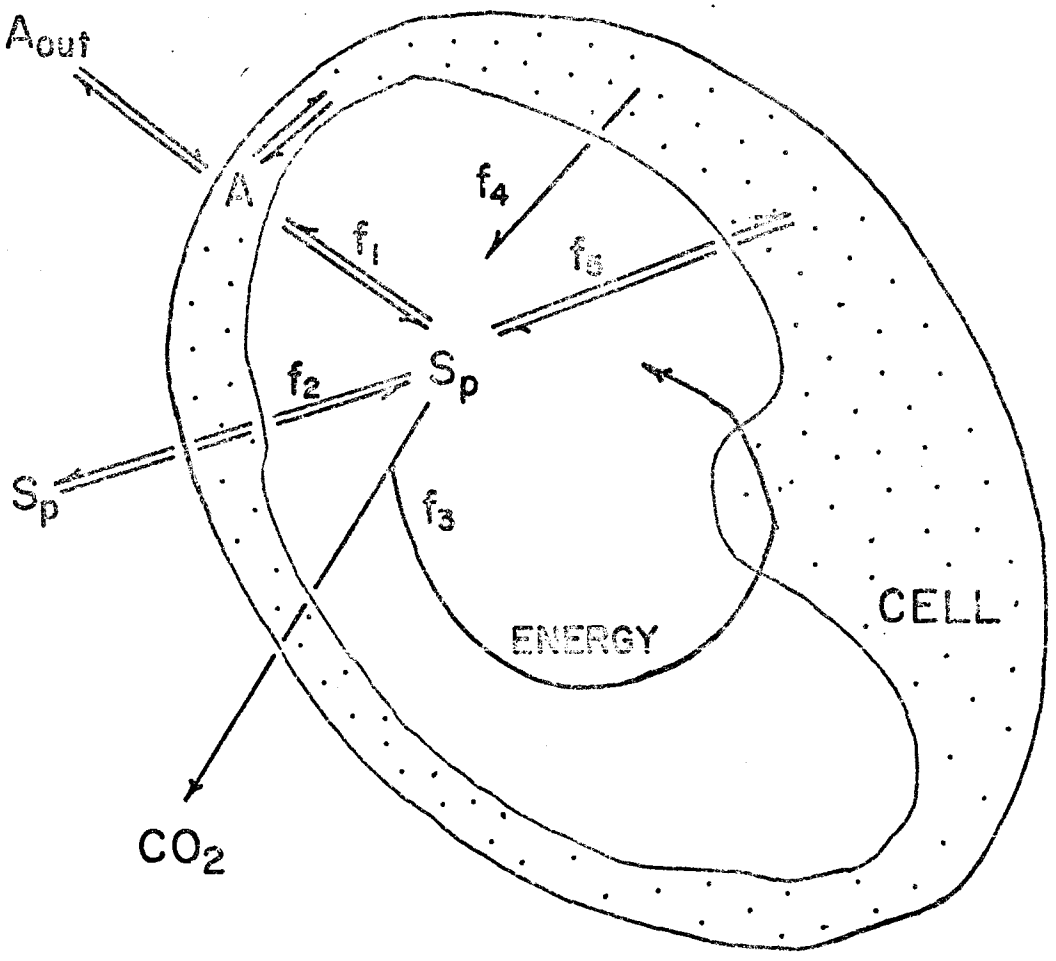

Figure 31. A simplified growth model based on the rates of intracellular soluble material formation and utilization.

V = reactor volume, liter

C_1 = constant

f_1 = the net flux of an intercellular substrate into the pool, g/hr

B. f_2, the rate of leakage of metabolic intermediates to the medium.

The leakage of metabolic intermediates from the pool to the medium is probably a natural consequence of the large concentration gradient that microorganisms generate across their envelopes by transport and metabolism. We assume, because of increased internal concentrations and increased transport rates involved with increased growth velocities, that the leakage rate is directly proportional to the growth rate.

$$f_2 = C_2 \mu X V \pi$$

where,

π = pool constituent leakage, g/g cells dry

C_2 = constant

f_2 = the net rate of metabolic intermediate leakage, g/hr

C. f_3, the rate of pool material consumption attending the supply of metabolic energy.

The energy required for cell growth processes is directly proportional to the growth rate; whereas the energy required for cell maintenance is assumed constant at all growth rates.

$$f_3 = K_3XV + C_3\mu S_p XV$$

where,

K_3 = rate of endogenous metabolism, g pool material/g cells/hr

C_3 = constant

f_3 = rate of consumption of pool material for providing energy, g/hr

D. f_4, the rate of turnover of macromolecules to the pool material.

This process is assumed directly proportional to the cell mass and the growth rate.

$$f_4 = (K_4 + C_4\mu)XV$$

where,

K_4 = rate of turnover of macromolecules to the pool material at zero growth rate, g pool material/g cells dry/hr

C_4 = constant

f_4 = rate of turnover of macromolecules into pool material, g/hr

E. f_5, the rate of utilization of pool material for biosynthesis.

Macromolecular biosynthesis for cell growth is assumed directly related to the intracellular soluble material concentration.

$$f_5 = K_\mu S_p X V$$

where,

K_μ = rate of incorporation of pool material for cell growth, hr^{-1}

f_5 = rate of utilization of pool material for macromolecule biosynthesis, g/hr

According the simplified growth model shown in Figure 31, the mass balance of intracellular soluble material concentration in a continuous culture can be stated as follows:

Rate of change of pool material in the reactor = Rate of input of pool material into the reactor − Rate of output of pool material from the reactor + Rate of accumulation of pool material in the reactor

$$V\frac{d(S_p X)}{dt} = 0 - FS_p X + (f_1 + f_4 - f_2 - f_3 - f_5) \quad .....(33)$$

where F is the flow rate of the medium, liter/hr. Dividing both sides of Equation (33) by V, we obtain

$$\frac{d(SpX)}{dt} = -rS_p X + (f_1 + f_4 - f_2 - f_3 - f_5)/V \quad .....(34)$$

where r is the dilution rate, hr^{-1}. At steady state, $d(S_pX)/dt = 0$; therefore Equation (34) becomes,

$$r\tilde{S}_p\tilde{X} = (f_1 + f_4 - f_2 - f_3 - f_5)/V \qquad \text{.....(35)}$$

where,

$\tilde{S}_p$ = steady state intracellular soluble material concentration, g/g cells dry

$\tilde{X}$ = steady state dry cell mass, g/l

Substituting the expressions of f_1, f_2, f_3, f_4 and f_5 into Equation (35), and letting $\mu = r$ [Equation (4)], Equation (35) becomes

$$\mu\tilde{S}_p\tilde{X} = C_1\mu\tilde{S}_p\tilde{X} + (K_4 + C_4\mu)\tilde{X} - C_2\mu\Pi\tilde{X} - K_3\tilde{X} - C_3\mu\tilde{S}_p\tilde{X} - K_\mu\tilde{S}_p\tilde{X} \qquad \text{.....(36)}$$

Dividing both sides of Equation (36) by $\tilde{X}$, and rearranging the equation,

$$\mu = \frac{K_\mu\tilde{S}_p + K_3 - K_4}{C_4 - C_2\Pi + (C_1 - C_3 - 1)\tilde{S}_p}$$

$$\mu = \frac{\bar{K}_\mu S_p + K_{3,4}}{C_{4,2} + \tilde{S}_p} \qquad \text{.....(37)}$$

where,

$$\bar{K}_\mu = K_\mu/(C_1 - C_3 - 1)$$

$$K_{3,4} = (K_3 - K_4)/(C_1 - C_3 - 1)$$

$$C_{4,2} = (C_4 - C_2\Pi)/(C_1 - C_3 - 1)$$

From experiments,

$$\mu \longrightarrow 0 \quad ; \quad \tilde{S}_p \longrightarrow \tilde{S}_{p,f}$$

$$\mu \longrightarrow \mu_{max}; \quad \tilde{S}_p \longrightarrow \tilde{S}_{p,max}$$

where,

$\tilde{S}_{p,f}$ = threshold intracellular soluble material concentration, g/g cells dry

$\tilde{S}_{p,max}$ = maximum intracellular soluble material concentration, g/g cells dry

As $\mu \rightarrow 0$, $\tilde{S}_p \longrightarrow \tilde{S}_{p,f}$, from Equation (37)

$$K_{3,4} = -\bar{K}_\mu \tilde{S}_{p,f} \qquad \text{.....(38)}$$

Substituting Equation (38) into Equation (37),

$$\mu = \frac{\bar{K}_\mu (\tilde{S}_p - \tilde{S}_{p,f})}{K_p + (\tilde{S}_p - \tilde{S}_{p,f})} \qquad \text{.....(39)}$$

where,

$$K_p = C_{4,2} + \tilde{S}_{p,f}$$

As $\mu \longrightarrow \mu_{max}$, $\tilde{S}_p \longrightarrow \tilde{S}_{p,max}$, and assuming $(\tilde{S}_{p,max} - \tilde{S}_{p,f}) \gg K_p$, Equation (40) becomes

$$\mu_{max} = \bar{K}_\mu \qquad \text{.....(41)}$$

Substituting Equation (41) into Equation (40), we obtain

$$\mu = \frac{\mu_{max} (\tilde{S}_p - \tilde{S}_{p,f})}{K_p + (\tilde{S}_p - \tilde{S}_{p,f})} \qquad \text{.....(42)}$$

Equation (42) predicts the response of intracellular soluble material concentration with respect to growth rate. This equation describes very well the experimental data obtained from arginine limited and arginine-glucose limited growth. The results are shown in Figure 32 where the data line was calculated based on Equation (42) with K_p = 0.0053 g/g cells dry, $\tilde{S}_{p,f}$ = 0.090 g/g cells dry and μ_{max} = 0.36 hr^{-1}. Equation (42) fails to address the relationship between the intracellular soluble material concentration and growth rate for glucose limited growth. While single substrate limited growth, particularly with glucose, seems normal to the microbiologists, it appears to be foreign to the organism. The organism does not seem to be well equipped for moderating metabolism with glucose concentration as the sole rate determinor. Thus the relationship between intracellular soluble material concentration and growth rate in glucose limited growth appears to be atypical.

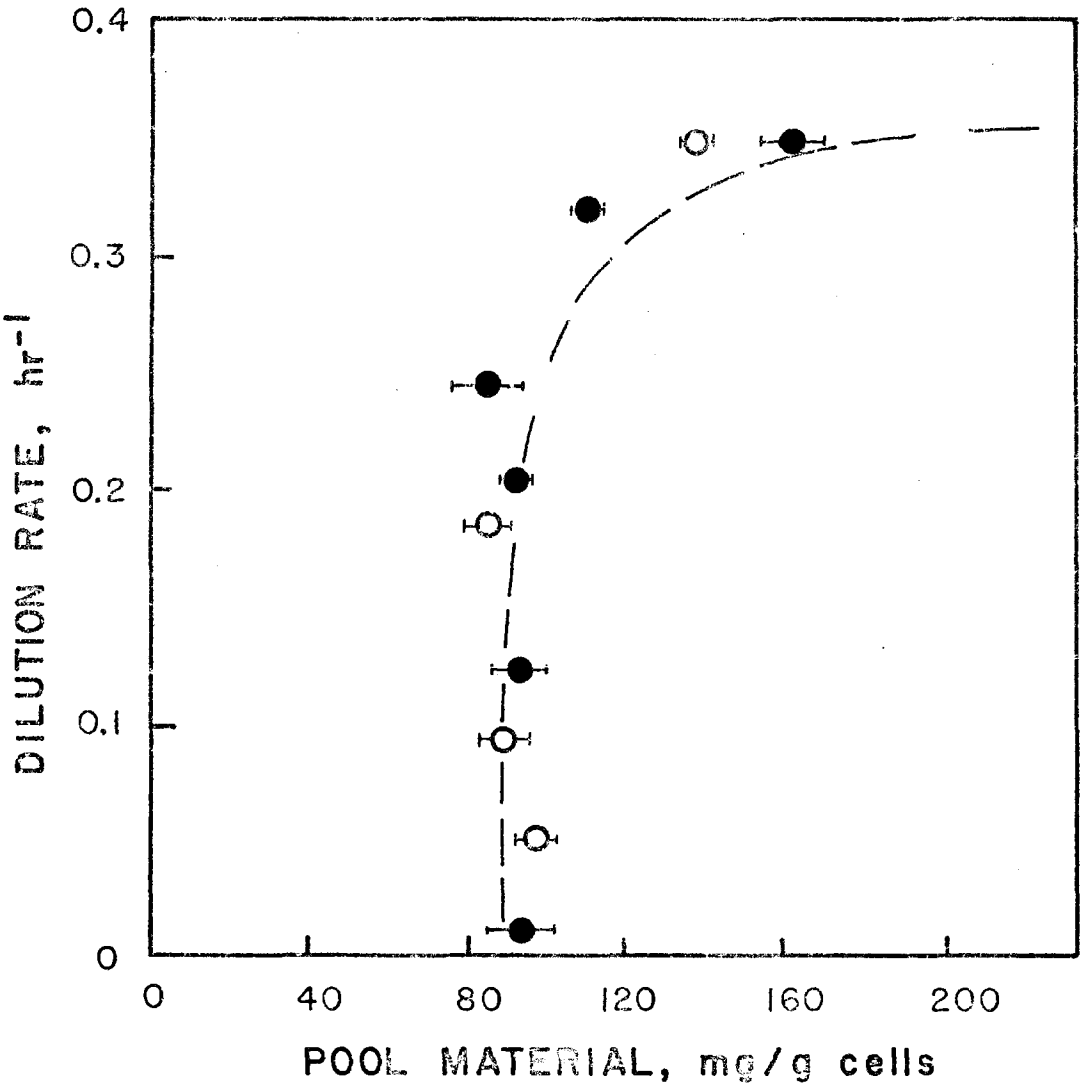

Figure 32. Relationship between intracellular soluble material concentration and growth rate. Data line was computed according to Equation (42).
Symbols: ● data obtained from arginine limited growth;
○ data obtained from arginine-glucose limited growth.

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