THE STABILITY AND SURVIVAL OF MIXED BACTERIAL CULTURES GENERATED IN A CHEMOSTAT

BY

WILLIAM GLEN RERIE

A Thesis

submitted to

the Faculty of Graduate Studies and Research

University of Manitoba

In partial fulfilment

of the requirements for the degree of

Master of Science

1977

THE STABILITY AND SURVIVAL OF MIXED BACTERIAL CULTURES GENERATED IN A CHEMOSTAT

by

William Glen Rerie

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

TO MY PARENTS

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. H. Halvorson, Professor of the Department of Microbiology, for his continued guidance and encouragement throughout the course of this research and in the preparation of this manuscript.

A special thanks to Mr. Gordon Moffatt for his assistance with the continuous culture apparatus and for the many helpful discussions, especially in the early stages of this study.

The friendship of the fellow graduate students of this department is also truly valued.

Financial assistance provided by a University of Manitoba graduate student fellowship is gratefully acknowledged.

ABSTRACT

ABSTRACT

Continuous culture was used in the growth of mixed bacterial cultures from a soil inoculum. Three media formulations were evaluated for their ability to generate a maximum number of different organisms under reasonable steady-state conditions. The complex media containing undefined growth factors permitted the simultaneous growth of up to eight bacterial types in the reactor, whereas the chemically-defined medium without growth factors allowed only three distinct species to be recognized.

Steady-state in the chemostats was monitored over a two or three week period using dry weight, total viable cell count and respiratory rate. Dry weight was shown to be a poor criterion of stability in the mixed population because of its apparent lack of sensitivity since it did not reflect oscillations in the population that were found to occur in the total viable count. During periods of biomass stability, the physiological parameter of respiratory rate of exogenous substrate may continue to fluctuate, suggesting internal changes in the microbial population. This concept was supported by data from the mixed culture grown on a chemically-defined medium. Increasing the dilution rate of this medium from 0.04 to 0.08 hr^{-1} had no affect on the number of organisms present or on the attainment of a better steady-state situation in the chemostat. Analyses of the residual carbon sources in the chemically-defined medium showed a consistency of the effluent substrate concentration only after a lengthy acclimatization period. The results demonstrate that steady-state as it is known in pure culture

studies, cannot be expected to be realized when growing heterogeneous cultures in a chemostat. Furthermore, 10 to 15 residence times may be required for mixed cultures to achieve their maximum stability.

Resting cell suspensions prepared from the mixed cultures were used to study starvation survival at 4^{o}, 20^{o} and $37^{o}C$. The parameter used to evaluate survival was found to be associated with the conclusions reached. Viable biomass as determined by the agar plating method underestimated survival in suspension. The measurement of cellular ATP was tested in one such instance and appeared to be a superior alternative for estimating viable biomass. The decline in endogenous respiration of the bacterial cells was not indicative of viability in the suspension whereas exogenous oxygen uptake in response to a multiple substrate proved to be a reliable parameter for assessing the physiological condition of the starved cells. On the basis of exogenous respiration the differences in the survival capabilities of the three culture sources were negligible. The loss of viability was either linear or curvilinear at 4^{o} and $20^{o}C$ but was exponential at $37^{o}C$.

The endogenous substrates available to the organisms were found to be influenced by their previous growth history. Cellular protein, carbohydrate and RNA were metabolized when the cultures had been grown in the complex media. The degradation of cellular RNA, however, occurred following the initial consumption of protein and respirable carbohydrate. Bacteria grown on the chemically-defined medium utilized protein and RNA but not carbohydrate and in this situation RNA was

degraded much more rapidly. The consumption of DNA was insignificant in all instances although loss of this component due to cellular leakage was observed in some cases.

TABLE OF CONTENTS

Table of Contents Cont'd PAGE

Table of Contents Cont'd

LIST OF FIGURES

List of Figures Cont'd

FIGURE PAGE

LIST OF TABLES

List of Tables Cont'd PAGE

PART I THE GROWTH AND STABILITY OF MIXED BACTERIAL CULTURES GENERATED IN A CHEMOSTAT

INTRODUCTION

INTRODUCTION

A number of studies carried out in this laboratory have relied on the use of mixed microbial populations obtained from a natural lagoon environment (2, 54). However inconsistencies in the composition of the population due to seasonal changes etc., injected an uncontrollable factor into the experimental work. It was therefore decided to explore the use of continuous culture to generate a mixed population of bacteria. This study was undertaken (a) to determine the number of different bacterial types that could be generated in a mixed culture and which medium formulation would permit the development of the maximum number of bacterial types; (b) to ascertain the degree of stability that is attained in the mixed cultures.

Three media formulations selected for the growth of mixed cultures in a chemostat environment were based upon previous history indicating their ability to meet the nutritional requirements of organisms indigenous to soil (48, 57, 93). Growth of bacteria under continuous culture conditions has been mathematically described in a number of early reports (41, 73, 96) and have been substantiated by pure culture experiments (41). More recently the use of continuous culture has been extended to the growth of multixenic cultures (19, 30, 34). In mixed microbial populations, however, interaction between species is known to occur (8, 72) which may disrupt steady-state as defined for pure cultures. Chian and Mateles (18) have stated that mixed cultures in a chemostat, require a longer period of acclimatization before reaching

a reasonable stability. Cassell et al., (16) have suggested heterogenous microbial populations represent a dynamic system which can never exhibit in a true steady-state. Therefore it was necessary to establish the extent of fluctuations in biomass occurring in the mixed cultures generated over a two-to-three week period of chemostat operation.

HISTORICAL

HISTORICAL

The continuous culture of microorganisms is a technique of increasing importance in all areas of microbiology. The principles and applications of continuous culture have contributed to advancements in the fields of bacterial physiology and genetics, technological and applied microbiology, and microbial ecology. The principal feature of this technique is that microbial growth occurs at a constant rate and in a stable environment. Factors such as pH, oxygen tension, concentration of nutrients and metabolic end products, which inevitably change during the growth cycle of a batch culture, are maintained constant in a continuous culture, which theoretically permits steady-state growth to proceed indefinitely.

In 1950 Monod (73) and Novick and Szilard (75) presented the first reports employing continuous dilution as a means of controlling the rate of bacterial growth. Since then their method has developed into the most common form of continuous culture, the chemostat. The apparatus consists of a culture vessel or reactor, which is fed sterile growth medium at a steady flow-rate and from which culture emerges at the same rate. The volume of the culture must remain fixed and the contents of the vessel sufficiently stirred to ensure that the entering growth medium is instantaneously and uniformly dispersed throughout the culture. The selection of a constant flow-rate (f) and culture volume (v) will impart upon the culture a specific dilution rate (D), defined as $D = f/v$, indicating the number of complete volume changes per unit time. The mean residence time (R) of a particle in the culture vessel is simply the reciprocal of the dilution

rate or 1/D = R.

If the environment of a bacterial population contains all the nutrients essential for growth, exponential growth will result according to equation

$$\frac{1}{x}\frac{dx}{dt} = \frac{\ln 2}{t_d} = \mu \qquad (1)$$

where x is the initial concentration of organisms, t_d is the culture doubling time and μ is the specific growth rate. The dependence of μ on substrate concentration(s) was shown by Monod (73) to be represented by the function

$$\mu = \mu_{max} \cdot \frac{s}{K_s + s} \qquad (2)$$

where the μ_{max} is the maximum growth rate when s is no longer growth-limiting, and K_s is a saturation constant which is numerically equal to the growth-limiting substrate concentration at 0.5 μ_{max}. In the chemostat the medium is compounded in such a way that all substrates required for growth of the organisms except one, are present in the culture at concentrations in excess of the growth requirement. Therefore growth rate of the population will be controlled by the availability of this single growth-limiting nutrient and it is the selection of the dilution rate which affords the experimenter this control.

Monod (73) and later Herbert et al., (41) presented a mathematical description of the influence of dilution rate on the kinetics of bacterial growth including its effect on the concentration of organisms

and growth-limiting substrate in the culture. In a reactor continually fed fresh nutrient, the growth of the culture is continuously offset by washout at a fixed dilution rate. The net change in concentration of organisms (x) will be determined by the relative rate of each process. Thus

$$\text{Increase} = \text{Growth} - \text{Output}$$

$$\frac{dx}{dt} = \mu x - Dx \qquad (3)$$

It follows that when $\mu > D$, dx/dt will be positive and the concentration of organisms in the culture will increase, while if $\mu < D$, dx/dt is negative and the concentration of organisms will decrease until eventually the culture "washes out" from the growth vessel. Only when $\mu = D$ will $dx/dt = 0$ and the concentration of organisms will remain constant with time. In this situation the culture is said to be in steady-state and therefore

$$\mu = \mu_{max} \cdot \frac{s}{K_s + s} = D \qquad (4)$$

It is not difficult to achieve steady-state with the chemostat since the growth rate of the organism is limited by the rate of supply of the growth-limiting nutrient to the culture. Therefore the specific growth rate will be directly proportional to dilution rate up to a critical value D_c where $D_c = \mu_{max}$. Dilution rates in excess of the maximum growth rate of the organism will result in its washout from the reactor according to equation 3.

Similarly, a mass balance equation can be constructed defining the net change in growth-limiting substrate concentration as a function of dilution rate.

$$\text{Change} = \text{Input} - \text{Output} - \text{Consumption}$$

$$ds/dt = DS_R - Ds - \text{Growth/Yield}$$

$$= DS_R - Ds - \mu x/Y \qquad (5)$$

where S_R and s represent the concentration of the growth-limiting component in the reservoir and reactor respectively and Y equals the yield factor (i.e. the weight of bacteria formed per unit weight of substrate consumed).

At steady-state when $dx/dt = 0$ and $ds/dt = 0$, unique values exist for both the biomass concentration ($\bar{x}$) and the concentration of residual substrate ($\bar{s}$) for each dilution rate. These values may be quantitatively described by the equations

$$\bar{s} = K_s \cdot \frac{D}{\mu_{max.} - D} \qquad (6)$$

and

$$\bar{x} = Y \ (S_R - s) \qquad (7)$$

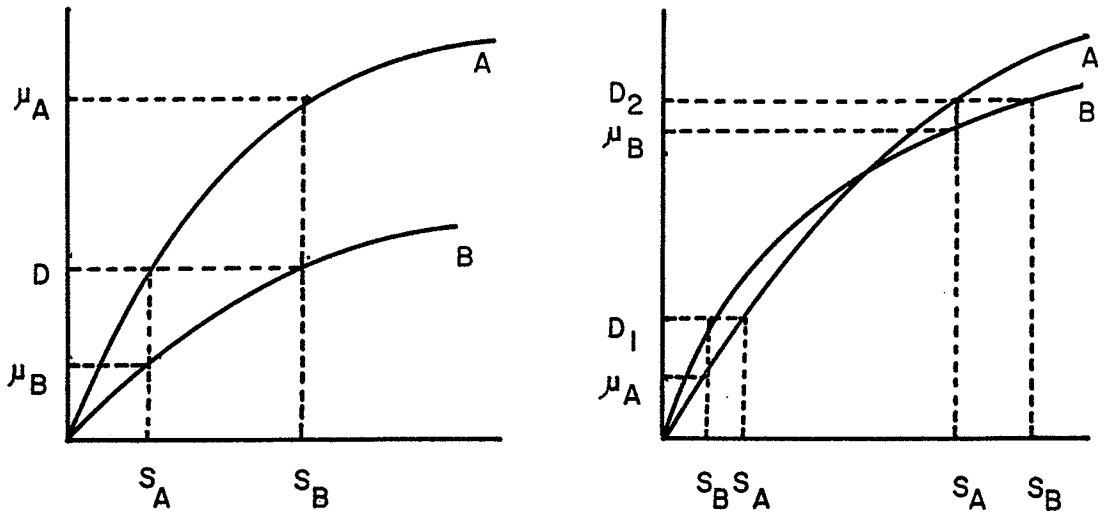
Equation 6 indicates that changes in dilution rate will alter the steady-state substrate concentration in the culture, thereby effecting a change in specific growth rate (eq'n. 2) and the doubling time of the organisms (eq'n. 1). The sole effect of varying the concentration of the growth-limiting nutrient (S_R) in the reservoir will be to change the steady-state microbial concentration (eq'n. 7), and will not alter

the residual substrate level or the specific growth rate of the organisms.

These equations quantitatively describe steady-state in a chemostat and have received experimental support in pure culture studies (34, 41). Nevertheless modifications to the basic theory have been proposed (59, 70) and reviewed by Tempest (96) and Pirt (80).

Within the last ten years the theory and applications of continuous culture have extended into the fields of environmental microbiology and microbial ecology where pure cultures have been replaced by the growth of mixed populations. The use of the chemostat for growing two-membered cultures is now widespread and the literature on the topic has become voluminous. Reviews on the subject have been presented by Bungay & Bungay (8), Meers (72), Jannasch & Mateles (52) and Veldkamp & Jannasch (102).

The steady-state composition of any mixed culture will depend upon the ability of the organisms present in the inoculum to become established in the chemostat under the fixed conditions of its operation. The enrichment of one organism over another can be predicted if the growth parameters μ_{max}, K_s and Y are known for each organism. The theoretical saturation curves depicted below are constructed using equation 2 for two organisms (A and B) having different K_s and μ_{max} constants. Referring to the figure on the left, it can be seen that when the organisms are grown in separate chemostats at a particular dilution rate (D), the growth-limiting substrate concentration would be s_A for organism A and s_B for organism B. Inoculating both organisms



into the same chemostat culture, operated at the same dilution rate, would result in organism A outgrowing organism B since at the growth-limiting substrate concentration s_A, organism B could only grow at the rate μ_B which, being less than D, would be insufficient to prevent it from being washed out of the culture. Regardless of the dilution rate chosen, organism A will displace organism B from the reactor. Competition of this form has been shown to occur between Escherichia coli and Azotobacter vinelandii (53). On the other hand, if the saturation curves of organisms A and B are like those shown in the figure on the right then the outcome of the competition will depend upon the dilution rate selected. At D_1 the substrate concentration at steady-state will be s_B, restricting organism A to grow at a rate of μ_A and because this growth rate is less than D_1, organism A will be eliminated from the culture. However, if D_2 is the selected dilution rate the competitive advantage between the two organisms is reversed with organism B now being diluted from the reactor as a consequence

of its growth rate falling below the dilution rate.

Meers (71) observed that the outcome of competition between Torula utilis and Bacillus subtilis in a magnesium-limited chemostat was controlled by the rate of dilution. Operated at 0.05 hr^{-1} the yeast outgrew the bacterium, whereas the opposite occurred at a dilution rate of 0.08 hr^{-1}. Jannash (50) has noted the inability of E. coli and Enterobacter to compete with marine organisms at low dilution rates for a single growth-limiting nutrient. Only when the dilution rate was increased to 0.5 hr^{-1} were the faster growing enterics able to become dominant in the culture. Therefore it is without doubt that dilution rate will be a major factor in determining the species composition of the culture that becomes established in the chemostat when inoculated with a heterogeneous population of organisms (49).

Enrichment of a mixed population in a chemostat need not result in the complete dominance of the culture by one species before steady-state conditions can be obtained. Taylor and Williams (95) outlined the mathematical theory of several species coexisting in a chemostat. They concluded that a stable mixed culture can exist only when the number of growth-limiting substrates present in the reactor is equal to or greater than the number of competing species. This statement, however, can only be true if there are no species interactions occurring within the mixed culture.

Support for this theory has been provided by Lewis (60) in growing yogurt starter strains of Lactobacillus and Streptococcus in mixed culture. The individual populations obtained from the mixed culture

were the same size as when grown separately, indicating each organism was limited by a different component in the medium. Titman (100) was able to grow a stable mixed population of two freshwater diatoms, Asterionella formosa and Cyclotella meneghiniana. The former was limited by phosphate while the latter was limited by silicate. Selection of the proper SiO_2:PO_4 nutrient ratio enabled both species to coexist in a single chemostat.

Unfortunately, prediction of the final steady-state composition of a mixed culture cannot always be made simply on the basis of the number of growth-limiting substrates supplied in the medium. The effect of microbial interactions developing between component members of the culture must also be considered. Bergter and Noack (3) grew Enterobacter cloacae under nitrogen limitation with excess glucose as the carbon and energy source. A second unnamed Gram-negative organism also used the same nitrogen source, but its growth was limited by organic acids released by E. cloacae. Continual oscillations in the mixed culture were observed in the reactor.

The release of a nutrient by an organism which limits the growth of a second member of the mixed culture was also reported in the continuous culture of Desulfovibrio desulfuricans with a Methanobacterium species (15). In this case D. desulfuricans growing on a lactate-limited medium released acetate as an end product of metabolism which in turn was consumed as the carbon source by the methane producer. This association could only persist if the Desulfovibrio was maintained

at a low population density since the release of hydrogen sulfide by the sulfate reducer was toxic towards the Methanobacterium.

Commensal relationships between organisms in a mixed culture may also occur when gorwth factors released by one species are necessary for the continued presence of a second species. Shindala et al., (87), found Proteus vulgaris was dependent upon Saccharomyces cerevisiae for the niacin that was excreted into the medium by the yeast. The ability of the slower-growing yeast to control the bacterial growth rate prevented the bacterium from displacing the yeast from the culture, allowing for a reasonably stable mixed culture to become established.

A mutualistic association involving growth factor release was shown to occur between Proteus vulgaris and Bacillus polymyxa (106). The Bacillus produced niacin required by P. vulgaris, which in turn provided B. polymyxa with biotin for growth. Sustained fluctuations in the ratio of the two organisms occurred because a second unidentified compound released by P. vulgaris was antagonistic towards B. polymyxa.

Chian and Mateles (18) isolated two colony types from a chemostat inoculated with polluted river water and identified them as a pseudomonad and a coliform species. In order to grow on the medium supplied, the pseudomonad was shown to require the presence of the coliform. When grown as a mixed culture, however, cell density varied from 10 to 20 percent even after 10-to-15 residence times. The preceding examples are indicative of the complexity of interspecies relationships that can arise in a chemostat and the resulting

destablizing affect they may have on the mixed culture.

The importance of these microbial interactions when examining natural biochemical processes in a chemostat was demonstrated by Munnecke and Hsieh (74) in a report studying the decontamination of parathion. The soil-or sewage-inoculated chemostat generated nine individual isolates, none of which was capable of growing on the insecticide alone. However, one of the isolates was capable of hydrolyzing parathion to p-nitrophenol, which in turn served as a utilizable substrate for a number of other organisms present in the mixed culture. Through the concerted action of the mixed culture, up to 50 mg of parathion per liter per hour was degraded.

Thus it is reasonable to assume that the establishment of a heterogeneous culture in a chemostat necessitates a certain degree of microbial interaction. What must be determined is whether or not these interactions disturb steady-state to an extent where reproducible data cannot be obtained due to continual oscillations in component species of the culture. Furthermore, the parameters used to evaluate steady-state in a mixed culture may affect the conclusions reached.

Much of the work describing the growth of heterogeneous populations in a chemostat has been performed with regard to waste water treatment, since the chemostat can be used to closely approximate the activated sludge process (29). In these studies the application of the kinetic equations of Monod was tested and their ability to describe steady-state growth of a mixed population under various operational conditions evaluated (19, 30, 32). Activated sludge inoculated into a glucose-

limited chemostat generated a mixed microbial population whose values for μ_{max}, K_s and Y changed as a function of dilution rate. In general, populations established at low dilution rates had low values of K_s and μ_{max} when compared to mixed cultures generated at high dilution rates. Ghosh and Pohland (32) explain that at low dilution rates organisms with high affinity for the substrate (i.e., low K_s) will have the selective advantage while as dilution rate increases those organisms with the shortest generation period (i.e. high μ_{max}) will become favoured. It was concluded that steady-state behavior of the system at a given dilution rate could be described by the equation $\mu = \mu_{max} \cdot s/(K_s + s)$ but the kinetic constants μ_{max}, K_s and yield factor cannot be considered true constants for such a population because of their dependence on the growth conditions selected.

Total biological solids and effluent Chemical Oxygen Demand (COD) are non-specific parameters often measured to ascertain steady-state behavior in complex systems. Gaudy and co-workers (30, 31) followed both parameters over a reasonable time period in a glucose-limited chemostat. Attainment of steady-state was realized with respect to effluent COD and effluent carbohydrate, but not with respect to total biological solids. Interestingly the effluent COD values were always greater than the corresponding values for effluent carbohydrate. Because glucose was the sole source of influent carbon, the difference between these values can be attributed to intermediary or end products of metabolism. The ability of mixed cultures to be

maintained on a single carbon-limited medium strongly suggests some of the organisms were existing on these metabolic end products and these inter-microbial associations were responsible for the instability of the total biological solids. Recently, Herbert et al. (43), have shown that extensive syntrophic interactions do occur between bacteria isolated from activated sludge.

Cassell et al.(16) have measured the levels of various alcohol-soluble pigments present in an activated sludge-inoculated chemostat which were used as indicators of the bacterial species present. They found that over a period of operation continual changes in the pigment composition of the mixed culture occurred, indicating fluctuations in the component members of the population. These results were confirmed by photomicrographs illustrating changes in the gross morphology of the culture during the experiment. A lack of stability was also recorded in the total biological solids and effluent COD.

In the assessment of heterogeneous microbial populations there is a noticeable absence of reports concerned with validating the parameters used to measure steady-state in the chemostat. Total biological solids, effluent COD and kinetic parameters are favored by engineers because of their lack of specificity (19, 30, 31, 34, 35, 97). From a microbiological viewpoint such gross parameters may lead to a false assurance that steady-state in a mixed culture has been attained. Knowledge of the frequency of interactions developing between individual components of a two-membered culture (8) and the destabilizing effect this may have on steady-state leads to the

assumption that when dealing with heterogeneous cultures such interactions are inevitable (16). It was therefore of interest to generate a mixed culture in a chemostat and assess its achievement of "steady-state" using several different parameters.

MATERIALS AND METHODS

MATERIALS AND METHODS

Description of the Chemostat

Throughout the course of this study, mixed bacterial populations were generated using the continuous culture apparatus developed by Kay (54) and shown in Fig. 1. The chemostat consisted of three basic components; the medium reservoir, the growth vessel and the effluent collection flask.

The continuous-flow growth vessel (A) was a modified 1-liter water-jacketed Bellco spinner flask (Bellco Glass Inc. Vineland, New Jersey). The glass dome was replaced by a plexiglass plate which was greased with high vacuum grease and clamped to the jar forming an air-tight seal. Three holes were drilled in each cover plate. The center hole received the shaft of the magnetic stirrer and was also connected to a cotton-filled filter serving as a sterile air inlet. One of the remaining two holes was used as an inlet port for the fresh medium, while the third hole, stoppered with a rubber ring, was used as a convenient sampling port. Fresh medium is fed from the carboy (E) to the growth flask by means of a MHRE/22/Delta multi-channel Watson-Marlow pump (D), (Fred A. Dungey, Agincourt, Ont.) The multi-channelled head enabled three independent chemostats to be operated simultaneously from a single pump. The influent rate of medium to each of the three cultures was checked daily by means of a flow meter (H) attached to each feed line. Deviations from the

Fig. 1 Continuous culture assembly

A - Water-jacketed reactor containing the mixed bacterial culture

B - Magnetic stirrer

C - Constant-temperature circulator

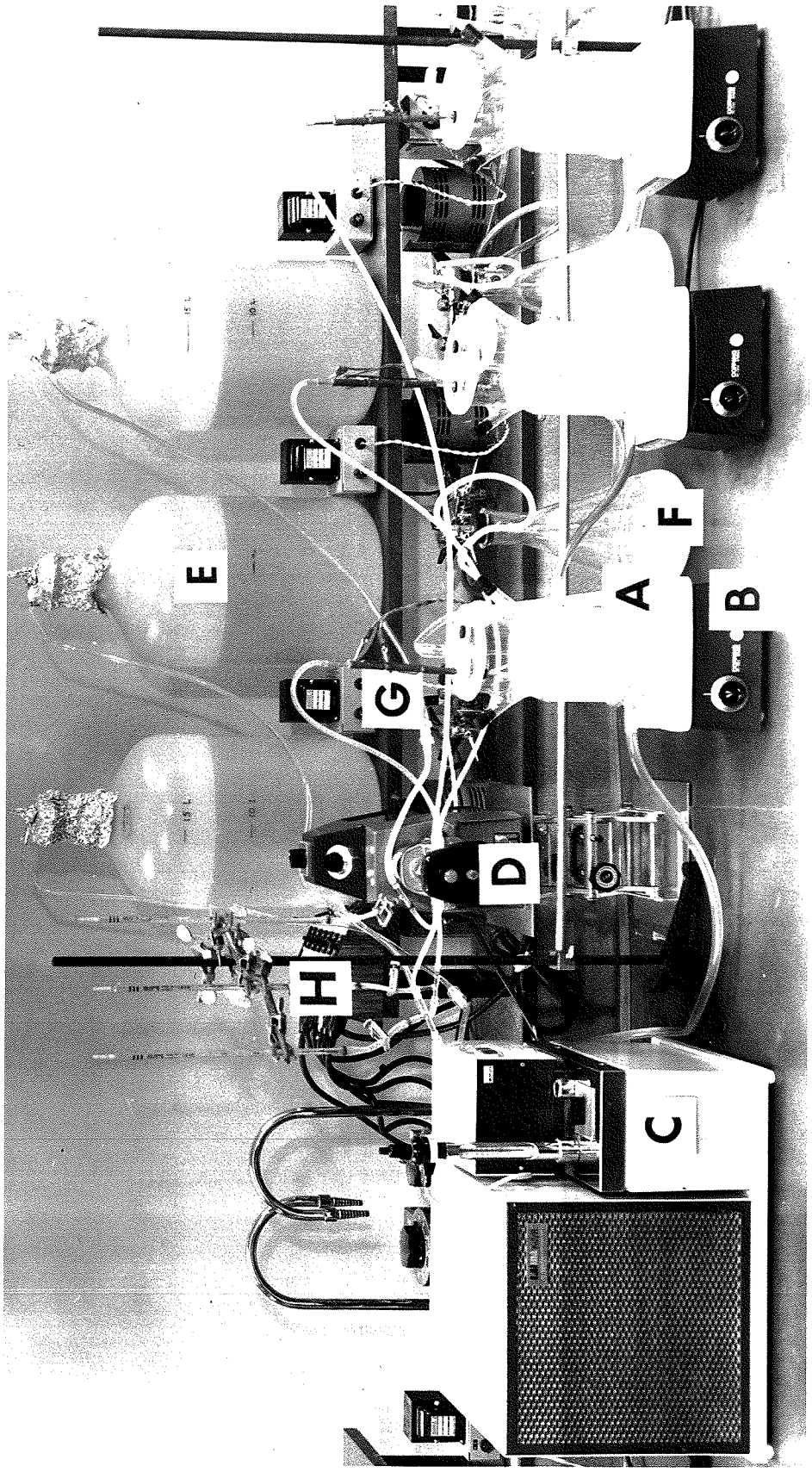
D - Watson-Marlow multi-channel pump

E - Sterile feed medium reservoir

F - Effluent collection flask

G - Porcelain-coated resistors surrounding steel tube leading into feed medium

H - Flow meter


A
B
C
D
E
F
G
H

desired rate of supply could be corrected by adjusting the pump speed directly or by altering the tension of the silicone tubing passing through the pump. In this manner each feed line could be controlled individually allowing the pump to supply 1-liter of nutrient to the culture every 25 hours.

In order to prevent bacterial contamination of the feed line, the final 20 cm of the line consisted of stainless steel tubing surrounded by three 10-ohm porcelain-coated resistors in series (G), joined to a step-down transformer. The transformer was in turn connected to an adjustable rheostat. A rheostat setting between 22 and 26 was sufficient to heat the influent medium over the 20 cm distance to a temperature of 55 - 60^{o}C at the flow rate of medium employed.

The culture volume was maintained at 1 liter by extending a piece of glass tubing into the growth flask through a side-arm. This effluent line was connected to a 2-liter vacuum flask (F) and when connected to the vacuum line, only a minimal amount of suction was required to draw excess culture from the growth vessel into the collection flask. This method not only kept the culture volume constant but also aided aeration by drawing air to the culture through the air filter connected to the top of the Bellco flask. Aeration was further enhanced by placing the culture vessel on a Corning PC-353 magnetic stirrer (B), (Corning Laboratory Products, Corning, N.Y.) supplying continuous vortex stirring to the culture. Temperature for growth was maintained at 20^{o}C $\pm$ 0.05^{o}C by a Neslab constant-temperature circulator (C), (Neslab Instruments Inc. Portsmouth, N.H.).

Inoculum for Mixed Culture

Bacteria indigenous to garden topsoil were used as the source of organisms for all mixed culture studies. A 10^{-4} dilution was prepared from 10 grams of soil using 0.1% sterile peptone water as the diluent. Ten ml of the above dilution was used to inoculate a liter of sterile medium for initial batch culture. Actidione (Calbiochem. San Diego, Calif.), an inhibitor of fungal growth, was added at this time to the starter culture to give a final concentration of 100 µg/ml. Within two or three days the batch culture had become established and the dilution could be initiated.

All but one of the experiments were conducted at a dilution rate of 0.04 $hr.^{-1}$. At this dilution rate the residence period (R) for a 1-liter culture is 25 hours. The remaining experiment was run at a dilution rate of 0.08 $hr.^{-1}$, accomplished by decreasing the culture volume to 500 ml. and thereby reducing the residence period to 12.5 hours.

Media

Two complex media and one chemically-defined medium were selected to evaluate their potential for generating mixed cultures of soil bacteria.

Bacto-peptonized milk medium: (Difco Laboratories, Detroit Mich). Peptonized Milk is an enzymatic digest of fresh skim milk. It contains hydrolytic products of the protein, albumins and globulins of milk. In this study it was rehydrated as described by Larkin (57).

Peptonized milk	15 gm
Double distilled water	15 liters
Final pH	6.5

Synthetic sewage medium: The artificial sewage medium of James (48) was used in place of natural sewage. Its components* are as follows:

Peptone (Bacto)	1.88 gm
Beef extract	3.75 gm
Dextrose	4.69 gm
$(NH_4)_2SO_4$	0.375 gm
NaCl	0.056 gm
KCl	0.0188 gm
$CaCl_2.2H_2O$	0.0413 gm
$MgSO_4.7H_2O$	0.188 gm
$NaH_2PO_4.H_2O$	19.808 gm
Na_2HPO_4	86.265 gm
Double distilled water	15 liters
pH adjusted to 7.4	

This formulation had a BOD_5 (20^oC) of 394 mg O_2 per liter.

*Analytical reagent grade chemicals were used.

Medium C

Medium C is the chemically-defined medium orginally outlined by Taylor (94) and later modified by Sundman and Carlberg (93). Its formulation* is as follows:

Glucose	7.5 gms
Sodium acetate	7.5 gms
Sodium lactate	7.5 gms
Sodium citrate	7.5 gms
Glycerol	7.5 gms
$(NH_4)_2HPO_4$	7.5 gms
K_2HPO_4	6.0 gms
$MgSO_4.7H_2O$	0.75 gms
NaCl	1.5 gms
$FeCl_3$	0.15 gms
Double distilled water	15 liters
Final pH adjusted to 6.8	

*Analytical reagent grade chemicals were used

All media, in 18-liter carboys, were autoclaved for 80 minutes at 121°C at 15 lbs. pressure.

Preparation of Resting Cell Suspensions

Resting cell suspensions of mixed bacterial populations to be used for manometric studies, were prepared in the following manner. Over a five-hour period, approximately 200 ml of culture was allowed to accumulate in a clean effluent collection flask. The cells were harvested by centrifugation in a model RC2-B Sorvall Superspeed centrifuge (Ivan Sorvall Inc. Norfolk, Conn.) operating at 10,000 rpm (16,300 xg) for 15 minutes. After centrifugation the supernatant liquid was discarded and the cell pellet was resuspended in 100 ml of 0.1M potassium phosphate buffer, pH 7.0. The washed suspension was spun down at 10,000 rpm for 15 minutes and the supernatant liquid decanted. The washed cell pellet was resuspended in 10 ml of phosphate buffer and allowed to stir vigorously for at least 30 minutes on a magnetic stirrer prior to use.

Manometric Measurement of Cell Respiration

Oxygen uptake was selected as a parameter to assess the physiological stability of the mixed cultures generated. A. Bronwill Warburg Respirometer (Bronwill Scientific Inc., Rochester, N.Y.) was used to measure respiration employing standard manometric techniques (101). The main compartment of the Warburg flask contained 2.0 ml of bacterial resting cell suspension along with 0.5 ml of 0.1M potassium phosphate buffer, pH 7.0. A small fluted filter paper and 0.2 ml of a 20% (w/v) KOH solution were placed in the center well to absorb CO_2 produced. One of the side-arms held 0.5 ml of a 0.75% (w/v) casamino acid solution (Difco Laboratories, Detroit, Mich.) serving as substrate.

Flasks were then attached to the manometers with their stopcocks open to the atmosphere, placed in the Warburg thermoregulated waterbath at 30°C, and shaken at a rate of 60 oscillations per minute. After a 20 minute period of temperature, gas and liquid equilibration, the stopcocks were closed and the substrate was tipped into the cell suspension. Manometer readings were taken at 10 minute intervals for a period of one hour. Data obtained were used to calculate the microliters of oxygen consumed in one hour by 1.0 milligram dry weight of cell suspension.

Dry Weight Determination

One method of assessing biomass stability of the mixed culture was by following changes in dry weight. To determine dry weight, a 5.0 ml sample taken from the culture flask was pipetted into a tared aluminum weighing dish and placed in a drying oven at 105°C overnight then weighed on a Mettler H 10T precision balance (Mettler Instruments, Zurich, Switzerland). The dry weight for each culture was then calculated as milligrams dry weight per ml of culture.

Viable Cell Determination

A second method used to determine the stability of the biomass in the mixed culture was to follow the total viable cell count during the operation of the chemostat. To determine the number of viable organisms present in the culture the spread plate method was used. A 1.0 ml sample of culture was serially diluted in 0.1% sterile peptone

water, and 0.1 ml samples of the appropriate dilution were evenly spread on duplicate plates of Trypticase Soy Agar (BBL, Cockeysville, Md.). The plates were incubated for 48 hours at 28°C in a Thelco incubator (Precision Scientific, Chicago, Ill.). After incubation, plates having between 30 and 300 colonies were counted and the number of viable organisms per ml of culture calculated.

The isolated colonies obtained by the above procedure were also used to determine the number of different soil organisms that were able to coexist in each of the three mixed cultures. Isolated colonies were picked off the plates and inoculated into sterile T-soy broth tubes and incubated at 28°C for three days. The broth culture was then restreaked on T-soy agar plates to ensure culture purity. Preliminary tests, (Tables 1 and 2) were carried out on each isolate, however no effort was made for complete identification of each organism.

Analyses of Residual Substrates in Medium C

The choice of a chemically defined medium allowed the measurement of the concentration of individual carbon sources present in the medium during the operational period of the chemostat. Both a 1-liter chemostat ($D = 0.04\ hr^{-1}$) and a 500 ml chemostat ($D = 0.08\ hr^{-1}$) fed with medium C were sampled regularly over a three-week period and the residual substrate levels of glucose, citrate, acetate, lactate and glycerol present in the culture were determined by the methods outlined below.

A 30 ml aliquot from each chemostat was centrifuged at 10,000

rpm (16,300 χg) for 15 minutes in a model RC2-B Sorvall Superspeed centrifuge (Ivan Sorvall Inc., Norfolk, Conn.). Approximately two-thirds of the supernatant fluid was decanted into a clean 25 x 150 mm screwcap test tube and stored frozen at -15°C until analyzed for each of the carbon components.

(i) Glucose

Glucose was quantitatively analyzed using the Worthington glucostat reagent set (Worthington Biochemical Corp. Freehold, N.J.) according to the semi-micro method outlined by Worthington. A series of 15-ml Corex centrifuge tubes was set up, one for each sample or standard, and 1.9 ml of distilled water was pipetted into each tube, followed by 0.1 ml of the respective sample or glucose standard. The tubes were mixed and 1.0 ml of 0.14N NaOH was added followed by 1.0 ml of a 2.0% $ZnSO_4$ solution. After mixing, the resulting precipitate was centrifuged down and 2.0 ml of the clear superantant liquid was pipetted into a clean 5" test tube. At timed intervals, 2.0 ml of the rehydrated Glucostat reagent was added to each tube, mixed, and allowed to stand at room temperature for exactly 10 minutes. One drop of 4.0N HCL was mixed into each tube at the end of the incubation period to stop the reaction. The tubes were allowed to stand at least five minutes before reading the absorbance at 420 nm.[1]

[1]All colorimetric measurements were made on a Beckman DB spectrophotometer (Beckman Instruments Inc. Fullerton, Calif.).

(ii) Citrate

Concentrations of sodium citrate in the medium were determined by coupling two reactions catalyzed by the enzymes citratase and malate dehydrogenase (104). Citrate is cleaved by the citratase into oxaloacetate and acetate. The oxaloacetate is in turn reduced to malate in the presence of malate dehydrogenase and NADH. The oxidation of NADH can be followed spectrophotometrically by observing the decrease in absorbance at 340 nm and is equivalent to the amount of citrate originally present in the sample.

Into a 3-ml cuvette the following reagents were added: 1.6 ml of 0.1M sodium phosphate buffer pH 7.5; 0.1 ml of 20 mM $MgCl_2 \cdot 6H_2O$; 1.0 ml of 1.0mM NADH; 0.1 ml malate dehydrogenase (125 Units/ml) and 0.1 ml of crude citratase. The cuvette was mixed and the initial absorbance at 340 nm was recorded. A volume of 0.1 ml of sample or citrate standard was then added to the cuvette, mixed, and the decrease in absorbance was measured. Absorbance readings were determined on a Gilford model 2400 recording spectrophotometer (Gilford Instruments, Oberlin, Ohio). Malate dehydrogenase and NADH were obtained from Sigma Chemical Co. (St. Louis, Mo.). The citratase was a crude extract provided by the department.

(iii) Acetate

Acetate was analyzed by gas chromatography using a Varian Aerograph Model 2100 gas chromatograph (Varian Aerograph, Walnut Creek, Calif.) equipped with a flame ionization detector. The flame was supported by a constant flow of hydrogen (35 cc/min) and compressed air (350 cc/min). The carrier gas was nitrogen supplied at 35 cc/min. Acetate was

separated by gas-solid chromatography in a 1.83 m by 4 mm internal diameter glass "U-shaped" column packed with Chromosorb 101, (Johns-Manville, Celite Div., Denver Co.). The injector and detector temperatures were maintained at 250°C, fifty degrees above the column temperature. The attenuation and range of the instrument were set at 128 and 10^{-12} respectively. A 10 µl syringe (Glenco Scientific Inc., Houston, Tex.) was used for the on-column injection of 5 µl samples or acetate standards.

Under these operational conditions acetate appeared as a single peak at 2.8 minutes after injection. The concentration of acetate was determined by evaluating the total area under the peak with the aid of a disc integrator.

(iv) Glycerol

Glycerol was determined by the method outlined by Burton (11). It is based upon the formation of formaldehyde as a product of glycerol oxidation by periodic acid. The formaldehyde formed is measured colorimetrically by the chromotropic acid-formaldehyde reaction.

(v) Lactate

The lactate content in the culture was estimated by the method described by Barker (1). Lactic acid is converted quantitatively into acetaldehyde on being heated in concentrated sulfuric acid. The acetaldehyde when reacted with p-phenylphenol forms a purple color compound. Lithium lactate was used to prepare all standards.

RESULTS

RESULTS

Generation of Mixed Cultures

Bacterial populations grown in the chemostat over a two-week period (~14 retention times), were screened to determined the number of different organisms that could coexist in the three growth media. Initial separation of colony types was based upon colony size, color and morphology. Some of the subsequent tests performed on the peptonized milk medium isolates and the synthetic sewage medium isolates are recorded in Tables 1 and 2 respectively. Over the operational period of the chemostat, eight distinctive colony types were obtained from the peptonized milk medium while seven colony types were isolated from the synthetic sewage medium. Both mixed cultures exhibited a predominance of Gram-negative, oxidase positive, motile rods. Only in the case of peptonized milk medium were Gram-positive, spore-forming rods (isolates 1 & 2) and oxidase negative, non-motile rods (isolate 7) obtained. The chemically-defined medium C supported the growth of three different organisms based upon colony size and appearance of which only two types predominated. No further biochemical tests were performed on the medium C isolates.

Respiratory Response to Casamino Acids by Mixed Cultures

The ability of each of the three mixed cultures to utilize casamino acids (CAA) as measured by oxygen uptake is shown in Fig. 2. The values

TABLE I Characteristics of the bacteria isolated from Peptonized Milk medium.

Culture No.	Morphology	Motility	Oxidase reaction	Gram reaction	Citrate utilization	Gelatin liquifaction	Nitrate reduction	Growth			Spore formation	Pigmentation
								Glucose	Sucrose	Arabinose		
1.2.	Rod	+	+	+	-	+	+	+	+	*	+	None
3.	Rod	+	+	-	+	+	+	+	+	+	-	Green
4.	Rod	+	+	-	+	-	-	+	-	-	-	None
5.8.	Rod	+	+	-	+	-	+	+	-	-	-	None
6.10.11.	Rod	+	+	-	-	-	-	+	-	-	-	Light Green
7.	Rod	-	-	-	+	-	+	-	-	-	-	White
9.	Rod	+	+	-	+	-	+	+	+	-	-	Buff
12.13.	Rod	+	+	-	+	-	-	+	-	+	-	Yellow

*Not tested

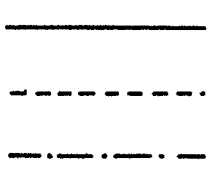
TABLE 2 Characteristics of the bacteria isolated from Synthetic Sewage medium.

Culture No.	Morphology	Motility	Oxidase reaction	Gram reaction	Citrate utilization	Urease	Gelatin liquification	Nitrate reduction	Growth			Pigmentation
									Glucose	Sucrose	Arabinose	
1.8.	Rod	+	+	-	-	-	-	-	+	-	+	light green
2.	Rod	+	+	-	+	-	+	+	+	+	+	light green
3.	Rod	+	+	-	+	-	+	+	+	+	+	white
4.	Rod	+	(+)	-	+	-	-	-	+	-	-	None
5.	Rod	+	+	-	+	+	+	+	+	*	*	Green
6.	Rod	+	+	-	+	-	-	+	+	-	-	None
7.9.	Rod	+	+	-	+	+	-	+	+	+	+	None

*Not tested

(+) Weak reaction

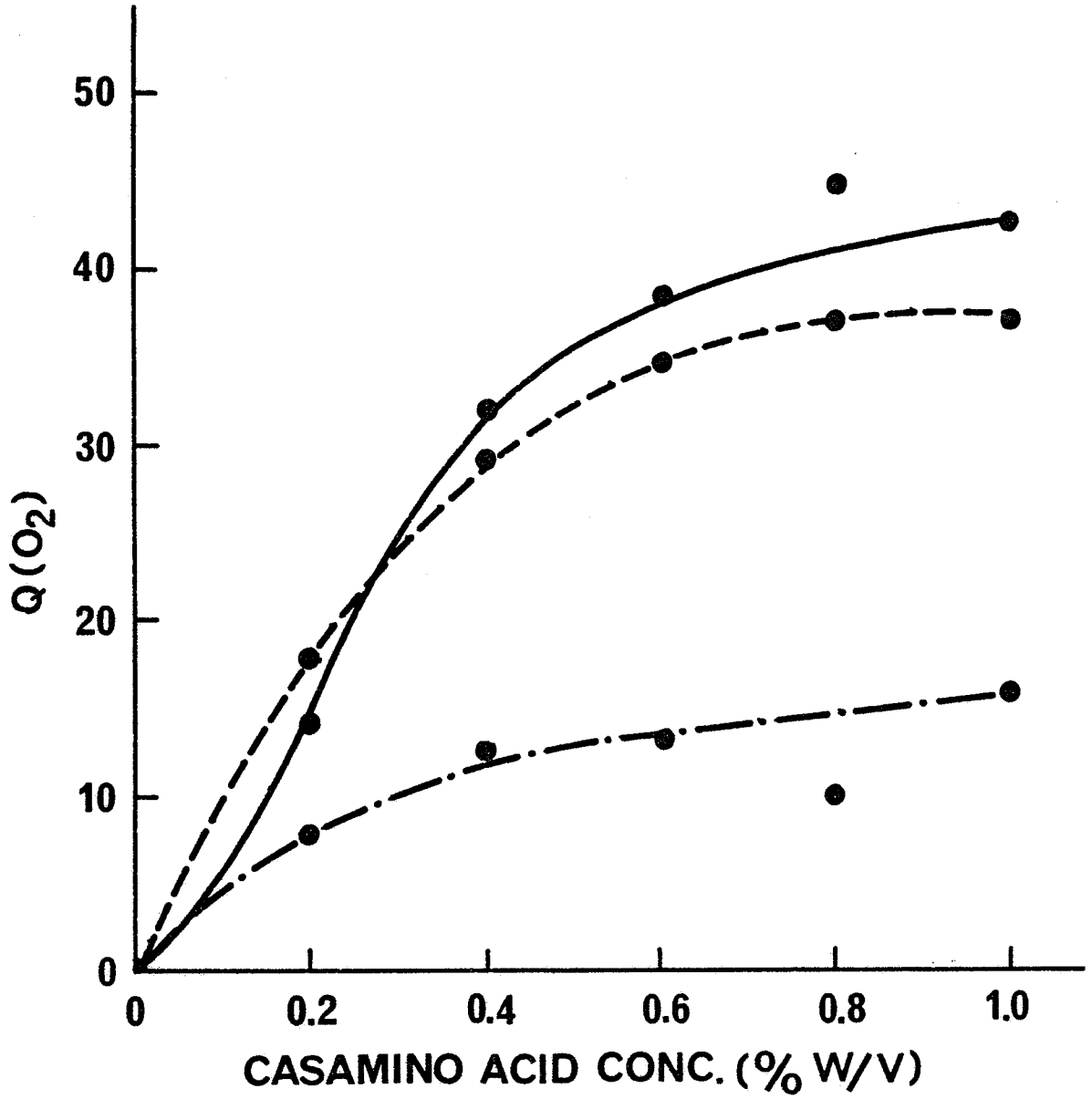
Fig. 2 Respiratory response to casamino acids by mixed cultures.



Peptonized milk culture

Synthetic sewage culture

Medium C culture


50
40
30
20
10
0
Q(O2)
0
0.2
0.4
0.6
0.8
1.0
CASAMINO ACID CONC. (% W/V)

are expressed as $Q(O_2)$ or $\mu l\ O_2 hr^{-1}$ mg dry weight^{-1}. On a milligram dry weight basis, the mixed cultures grown on peptonized milk medium and synthetic sewage medium showed a similar response to the casamino acids, whereas medium C-grown cultures exhibited significantly lower rates of oxygen uptake. At 0.75% CAA, the substrate concentration used in subsequent manometric experiments, the calculated $Q(O_2)$ values were 40.5, 37.0 and 14.5 for peptonized milk, synthetic sewage and medium C-grown cultures respectively.

Figure 2 also indicates that 0.5 ml of a 0.75% casamino acids solution was sufficient to saturate the cell suspension over the entire one hour incubation period, thereby not limiting the amount of oxygen consumed.

Physiological and Biomass Stability of Mixed Cultures

Oxygen uptake, dry weight and viable cell count were used to measure the physiological and biomass stability in the mixed culture during the operation of the chemostat. The three parameters were monitored for 12 retention periods in cultures generated on peptonized milk medium and synthetic sewage medium and over 20 retention periods in the medium C culture. The chemostats were operated at a dilution rate of 0.04 hr^{-1} (R = 25 hours). The results are shown in Figs. 3, 4 and 5.

Upon initiating the dilution, it is generally assumed that four to six turnover periods are required to establish steady-state conditions in pure culture. Our results showed that the dry weight of the cultures declined initially but reached a relatively constant value by the fourth

Fig. 3 Stability of the mixed culture grown on peptonized milk medium at D = 0.04 hr^{-1}.

—·—·—·— $Q(O_2)$

– – – – – – – – Log viable cell count

———————— Dry weight

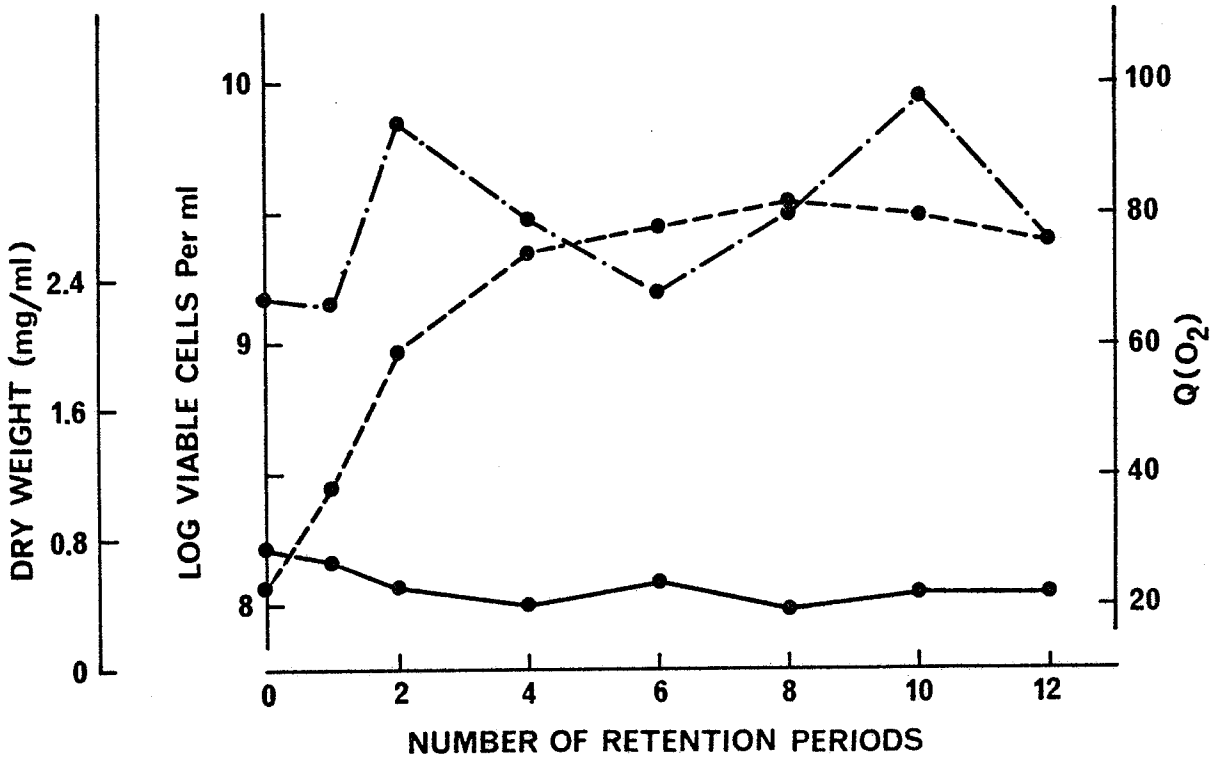

DRY WEIGHT (mg/ml)
2.4
1.6
0.8
0
LOG VIABLE CELLS Per ml
10
9
8
100
80
60
40
20
Q(O2)
0
2
4
6
8
10
12
NUMBER OF RETENTION PERIODS

Fig. 4 Stability of the mixed culture grown on synthetic sewage medium at D = 0.04 hr^{-1}.

—·—·—·— $Q(O_2)$

- - - - - - - - - Log viable cell count

————————— Dry weight

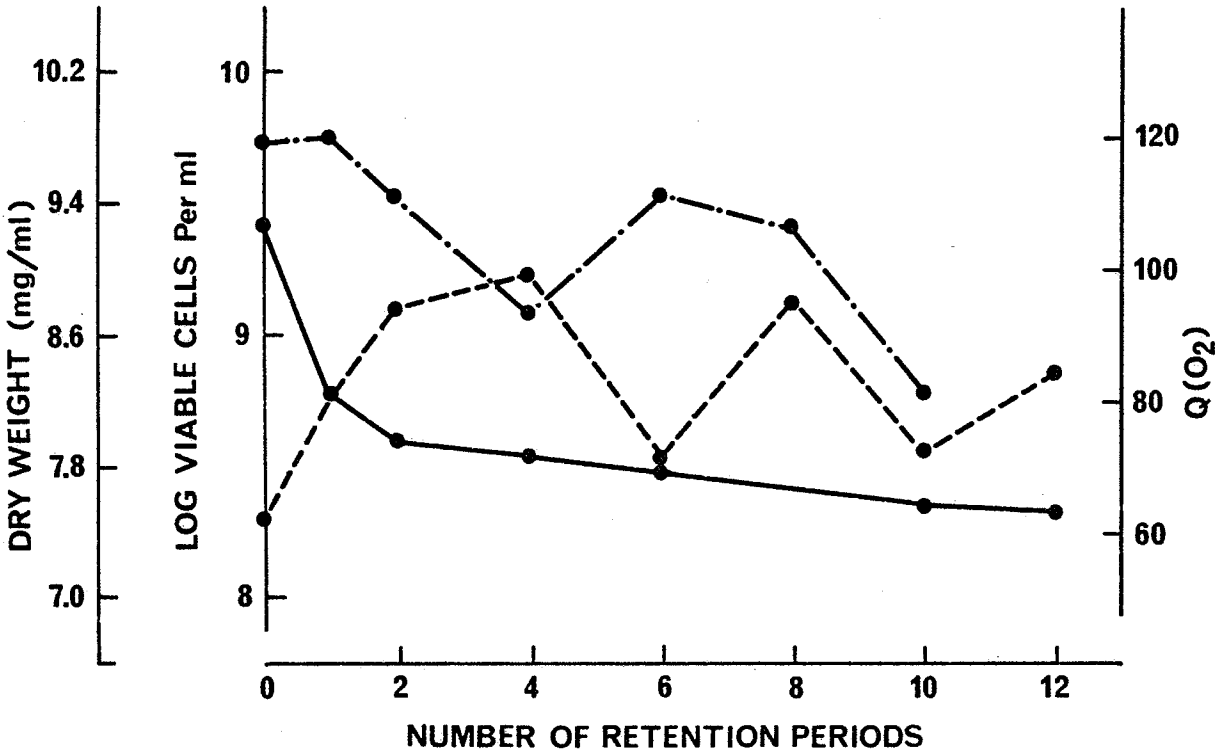
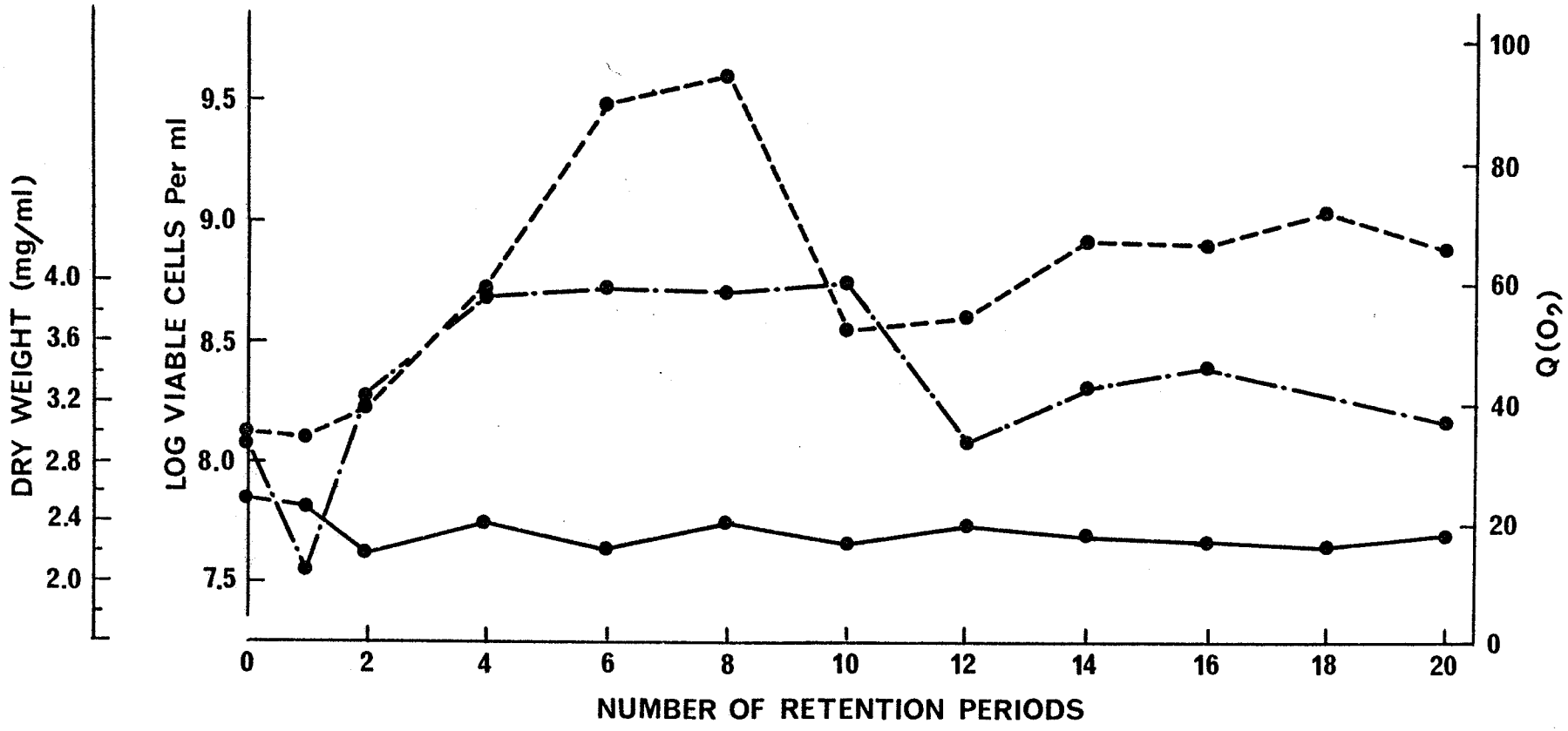

DRY WEIGHT (mg/ml)
10.2
9.4
8.6
7.8
7.0
LOG VIABLE CELLS Per ml
10
9
8
Q(O2)
120
100
80
60
0
2
4
6
8
10
12
NUMBER OF RETENTION PERIODS

Fig. 5 Stability of the mixed culture grown on medium C at D = 0.04 hr^{-1}.

—·—·—·— $Q(O_2)$

- - - - - - - - Log viable cell count

———————— Dry weight


DRY WEIGHT (mg/ml)
4.0
3.6
3.2
2.8
2.4
2.0
LOG VIABLE CELLS Per ml
9.5
9.0
8.5
8.0
7.5
Q(O2)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
NUMBER OF RETENTION PERIODS

retention period. Contrary to dry weight, the viable cell counts increased substantially during this time. In the peptonized milk culture the total viable count suggested stability between R4 and R12 with counts of 3.0×10^9 cells/ml. The synthetic sewage and medium C mixed cultures, however, continued to exhibit viable biomass fluctuations throughout the twelve retention periods. During this time viable counts ranged from $0.34 - 1.68 \times 10^9$ cells/ml in the synthetic sewage culture and from $0.36 - 3.98 \times 10^9$ cells/ml in the medium C culture.

The respiratory response of the mixed cultures to the CAA substrate indicated changes were also occurring in this physiological parameter. Even in the mixed culture generated on peptonized milk medium the $Q(O_2)$ showed some oscillation, although between R4 and R12 values averaged from 75 to 80.

In the case of the mixed culture grown on medium C, data were collected over a further eight retention periods to a final total of twenty. As seen in Fig. 5, between R12 and R20, reasonable steady-state conditions were obtained as shown by the stability of all three parameters. The culture dry weight remained at 2.35 mg/ml and the viable cell count equalled 7.92×10^8 cells/ml with a $Q(O_2)$ value of approximately 40 during this interval.

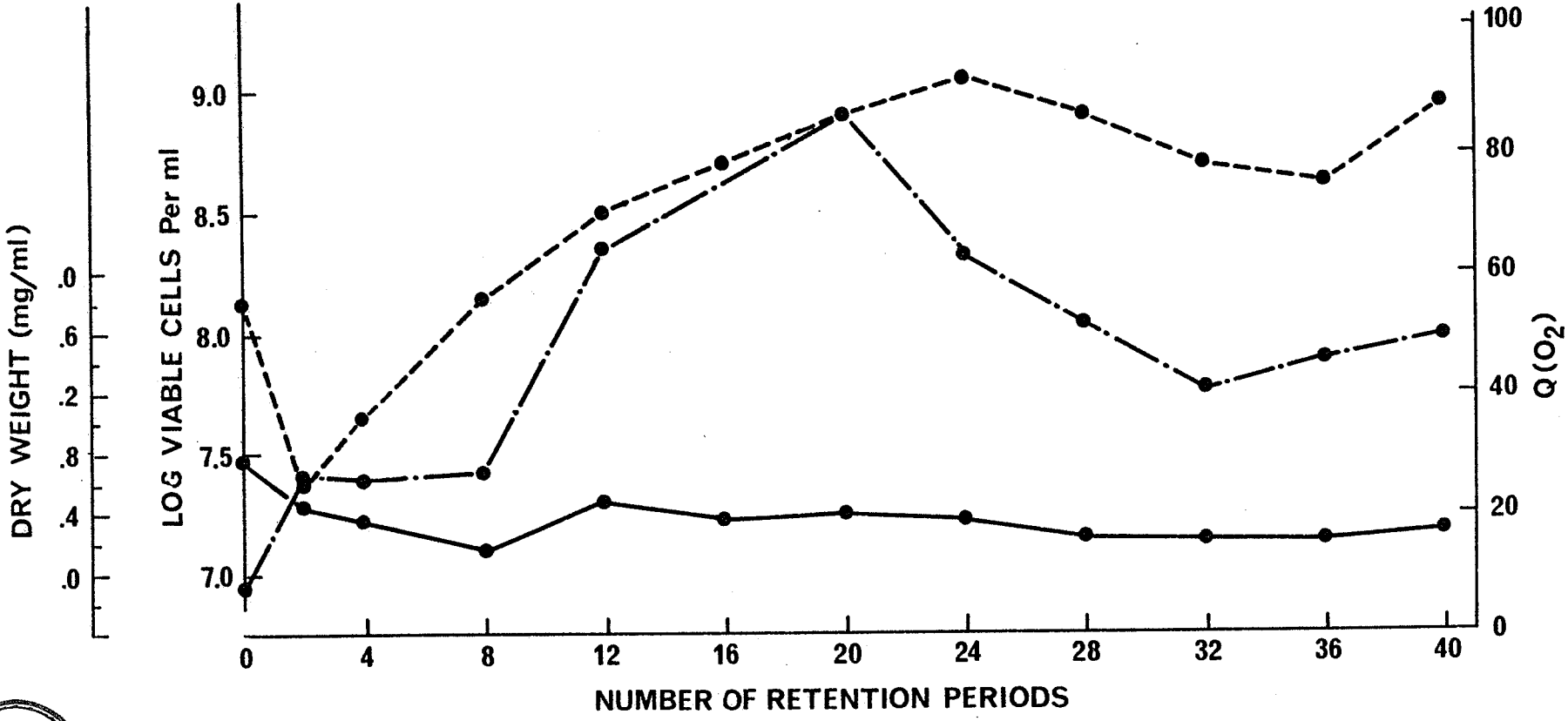
To determine if a faster dilution rate would affect the time required to obtain a stable condition in the medium C mixed culture the flow rate was increased to $D = 0.08\ hr^{-1}$, thereby reducing the retention time to 12.5 hours. The results for this mixed culture are shown

Fig. 6 Stability of the mixed culture grown on medium C at D = 0.08 hr^{-1}.

—·—·—·— $Q(O_2)$

---------- Log viable cell count

———————— Dry weight



in Fig. 6. The faster dilution rate did not markedly alter the results previously observed for the medium C culture. By R20 and through R40 the mixed culture displayed a dry weight of 2.3 - 2.4 mg/ml with a viable cell count between 0.43 and 1.12 x 10^9 cells/ml. suggesting biomass stability. However, the physiological parameter of $Q(O_2)$ fluctuated significantly within this period, falling from 86 at R20 to 41 at R32.

Viable Count of Individual Isolates from the Medium C Mixed Culture

The ability of medium C to support the growth of only three different organisms that were readily distinguishable on T-soy agar permitted the enumeration of each of the isolates along with the total viable count. The results for the mixed cultures obtained at dilution rates of 0.04 and 0.08 hr^{-1} are shown in Tables 3 and 4 respectively. At both dilution rates the same three organisms became established in the culture. Isolate A grew quite slowly on T-soy agar and after 48 hours incubation appeared as a small translucent colony. Isolate B however grew rapidly, forming large shiny cream-colored colonies. The third organism isolated from the culture grew well on T-soy agar and produced a characteristic yellow-pigmented colony.

Operating at a diluton rate of 0.04 the medium supported a total population that varied from 3.6 x 10^8 to 3.9 x 10^9 cells/ml. Prior to the start of the dilution isolate B was found to be the predominant organism in the culture. However, within four retention periods a substantial shift in the population towards the establishment of isolate A had

occurred and by the eighth retention period it outnumbered isolate B by a ratio of 40 to one. The high cell count of organism A at R8 appears only as a transient state since its viable count declined from 3.8×10^9 to 1.2×10^8 cells/ml by R10 and allowed organism B to regain a slight dominance in the culture. The following eight retention periods, R10 to R18, again indicated a steady increase in the numbers of organism A to a maximum of 9.8×10^8 cells/ml. Isolate C generally remained below 10^5 cells/ml throughout the operation of the chemostat and therefore its actual numbers were not determined.

The results in Table 4 demonstrate that the increased dilution rate had a minimal effect on controlling fluctuations in the total population. It did however seem to reduce the degree of variation in the viable counts of organism B. Through 28 retention periods, from R12 to R40, the viable count of organism B varied from 1.0 to 2.5×10^8 cells/ml. The stabilization of isolate B in the mixed culture resulted in a moderate decrease in the range of fluctuation of organism A to organism B. Isolate C was again a minor component of the population, although its numbers were slightly more evident at the higher dilution.

The preceding tables also indicate that while the total viable cell count in the reactor can be similar at two separate intervals the species composition of the mixed culture can be quite different. For example, at R14 and R16 (Table 3) the number of viable cells was determined to be 8.5 and 8.2×10^8 respectively. When assessing the viable counts for the individual isolates the data indicated a significant shift in composition of the population with the ratio of isolate A to isolate B having increased from five at R14 to 13 at R16. Similar data can be seen at R20 and R28 in Table 4.

TABLE 3 Viable cell count of individual isolates from the Medium C mixed culture at D = 0.04 hr^{-1}.

No. retention periods elapsed	Viable cell count (Cells/ml x 10^8)				Ratio
	Total	A*	B**	C***	A:B
0	1.4	<1.0	1.0	-	-
4	5.2	5.0	<1.0	$1x10^5$	-
8	39.0	38.0	1.0	-	40
10	3.6	1.2	2.4	-	0.5
12	4.1	1.6	2.5	-	0.7
14	8.5	7.1	1.4	-	5
16	8.2	7.6	0.6	-	13
18	11.0	9.8	1.2	-	8
20	7.8	5.9	1.9	-	3

* Isolate A: small transparent colony on T-soy agar.

** Isolate B: large opaque colony on T-soy agar.

*** Isolate C: yellow-pigmented colony on T-soy agar.

TABLE 4 Viable cell count of individual isolates from the Medium C mixed culture at D = 0.08 hr^{-1}.

No. retention periods elapsed	Viable cell count (Cells/ml x 10^8)				Ratio
	Total	A*	B**	C***	A:B
0	1.4	<1.0	1.0	-	-
12	3.2	1.0	2.2	1 x 10^6	0.5
16	5.0	2.5	2.5	1 x 10^5	1
20	8.2	8.0	1.0	-	8
24	12.0	10.0	1.0	-	10
28	8.0	6.5	1.5	-	4
32	5.0	3.4	1.6	1 x 10^5	2
36	4.3	3.0	1.0	-	3
40	7.9	6.0	2.0	-	3

* Isolate A: small transparent colony on T-soy agar.

** Isolate B; large opaque colony on T-soy agar.

*** Isolate C; yellow-pigmented colony on T-soy agar.

Standard Curves for Quantitative Analysis

Standard curves required for the quantitative measurement of lactate, glucose, and glycerol are given in Fig. 7. The standard curves for citrate and acetate are shown in Figs. 8 and 9 respectively.

Concentration of Residual Substrates in Medium C

The chemically-defined formulation of medium C permitted the monitoring of individual carbon sources available to the mixed culture during the operation of the chemostat. Samples taken over 20 retention periods at D = 0.04 hr^{-1} and over 40 retention periods at D = 0.08 hr^{-1} were analyzed for residual levels of glucose, citrate, acetate, lactate and glycerol. Figs. 10 - 14 illustrate the results obtained. All substrates, except lactate, were fed to the culture at a concentration of 0.5 mg/ml of medium. Lactate was present at 0.25 mg/ml.

It is immediately apparent from the figures that the organisms comprising the mixed culture selectively assimilated certain substrates such as glucose and glycerol in preference to other compounds namely acetate and lactate. Furthermore the slower diluton rate facilitated a more efficient utilization of the substrates present in the medium.

Fig. 10 shows that upon initiating the dilution at a rate of 0.04 hr^{-1}, residual glucose in the culture rapidly reached a steady-state concentration of 15 µg/ml. Increasing the dilution rate to 0.08 hr^{-1} effected only to delay the attainment of a steady-state condition with respect to glucose. In the time of the first four retention periods the

Fig. 7 Standard curves for the quantitative analysis of

—·—·—·— Lactic acid @ 570 nm

———————— Glucose @ 570 nm

- - - - - - - - - Glycerol @ 420 nm

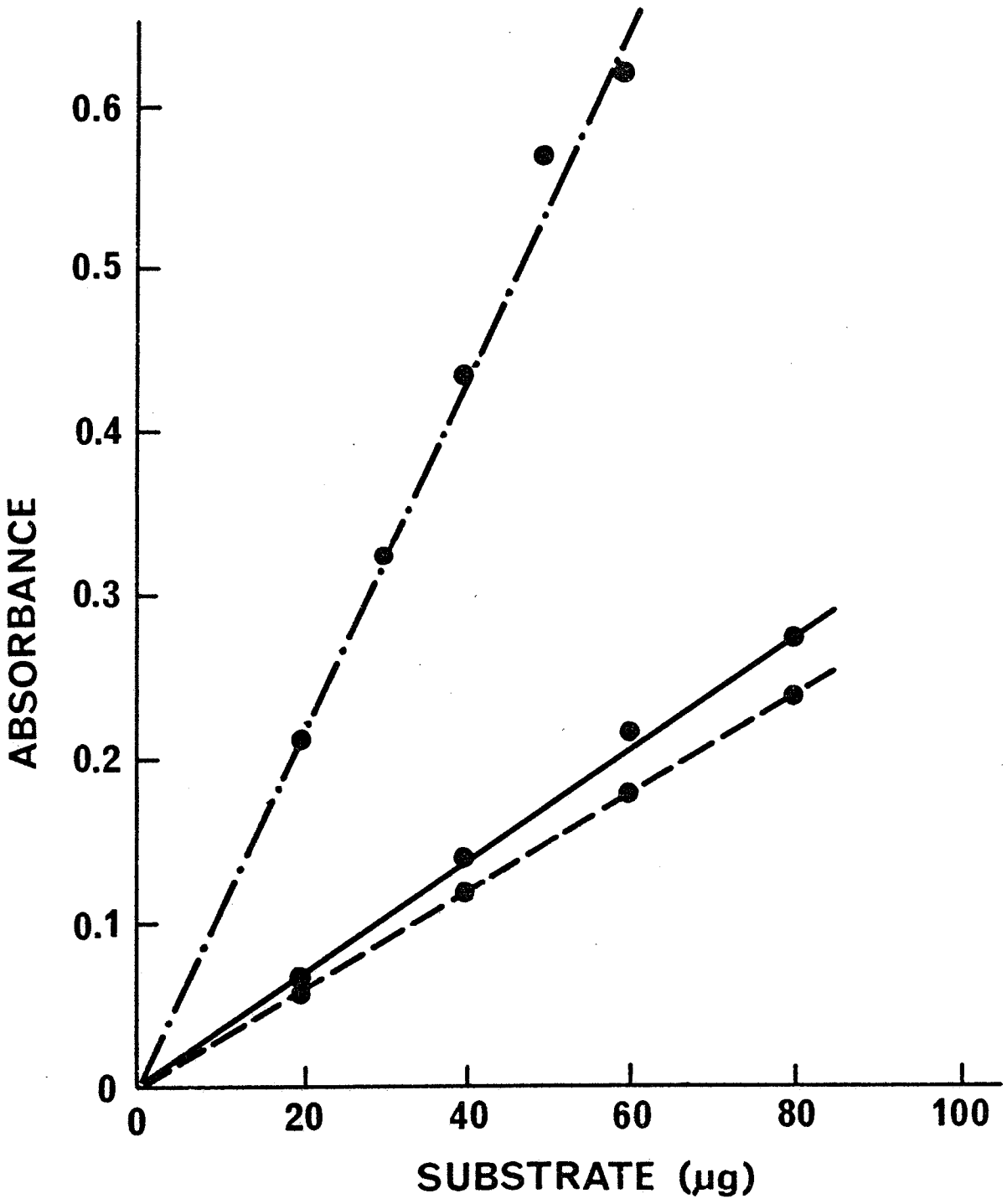
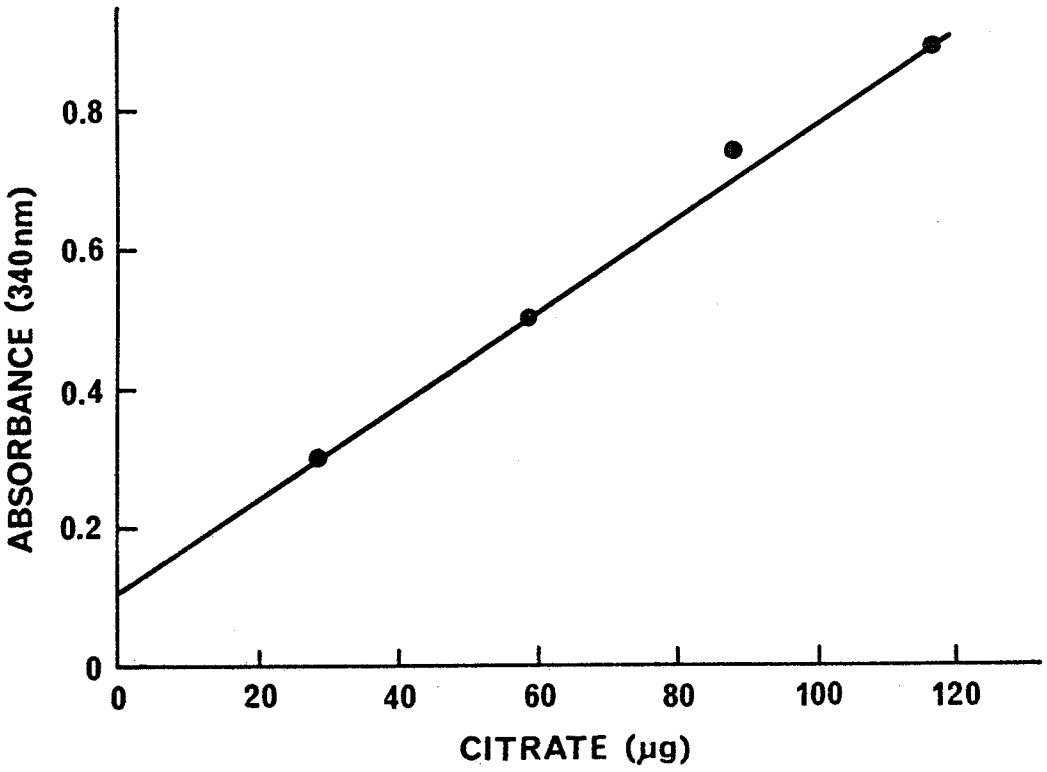

ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
SUBSTRATE (µg)

Fig. 8 Standard curve for the quantitative analysis of citrate.

Fig. 9 Standard curve for the quantitative analysis of acetate.


ABSORBANCE (340nm)
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
CITRATE (µg)

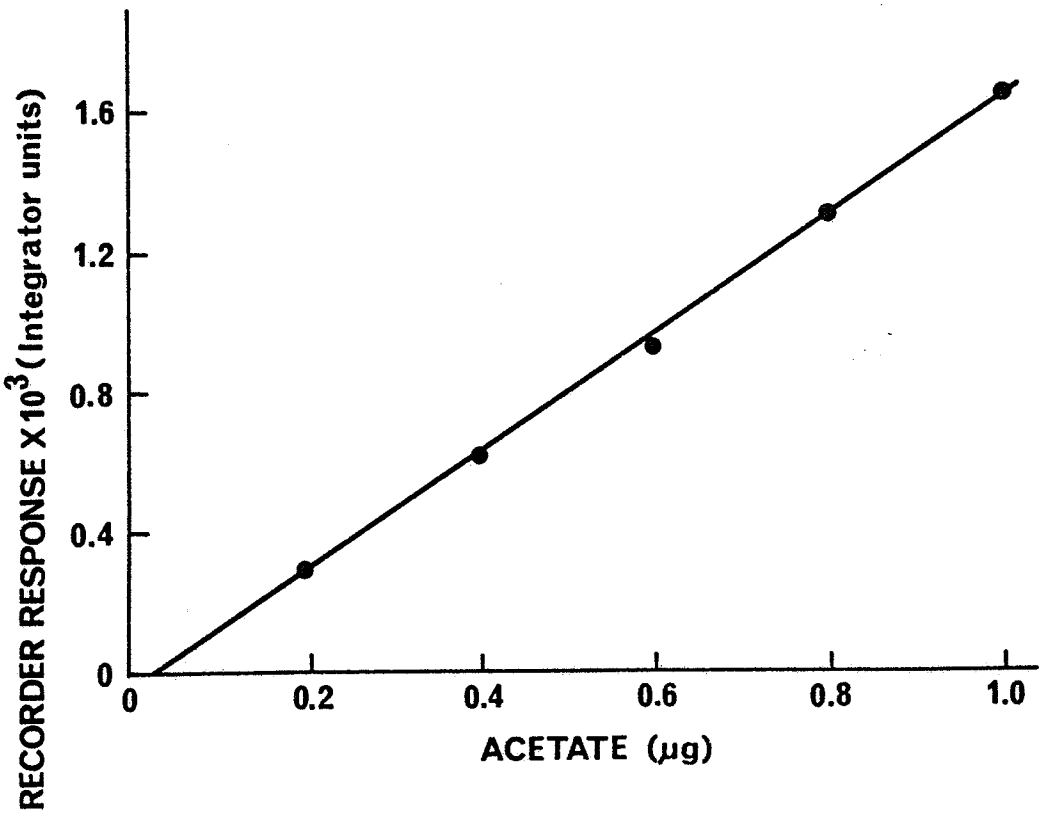

RECORDER RESPONSE X10^3(Integrator units)
1.6
1.2
0.8
0.4
0
0
0.2
0.4
0.6
0.8
1.0
ACETATE (µg)

Fig. 10 Concentration of residual glucose in the medium C mixed culture.

———— D = 0.04 hr^{-1}

- - - - - - - - - - D = 0.08 hr^{-1}

Fig. 11 Concentration of residual citrate in the medium C mixed culture.

———— D = 0.04 hr^{-1}

. . . . . D = 0.08 hr^{-1}

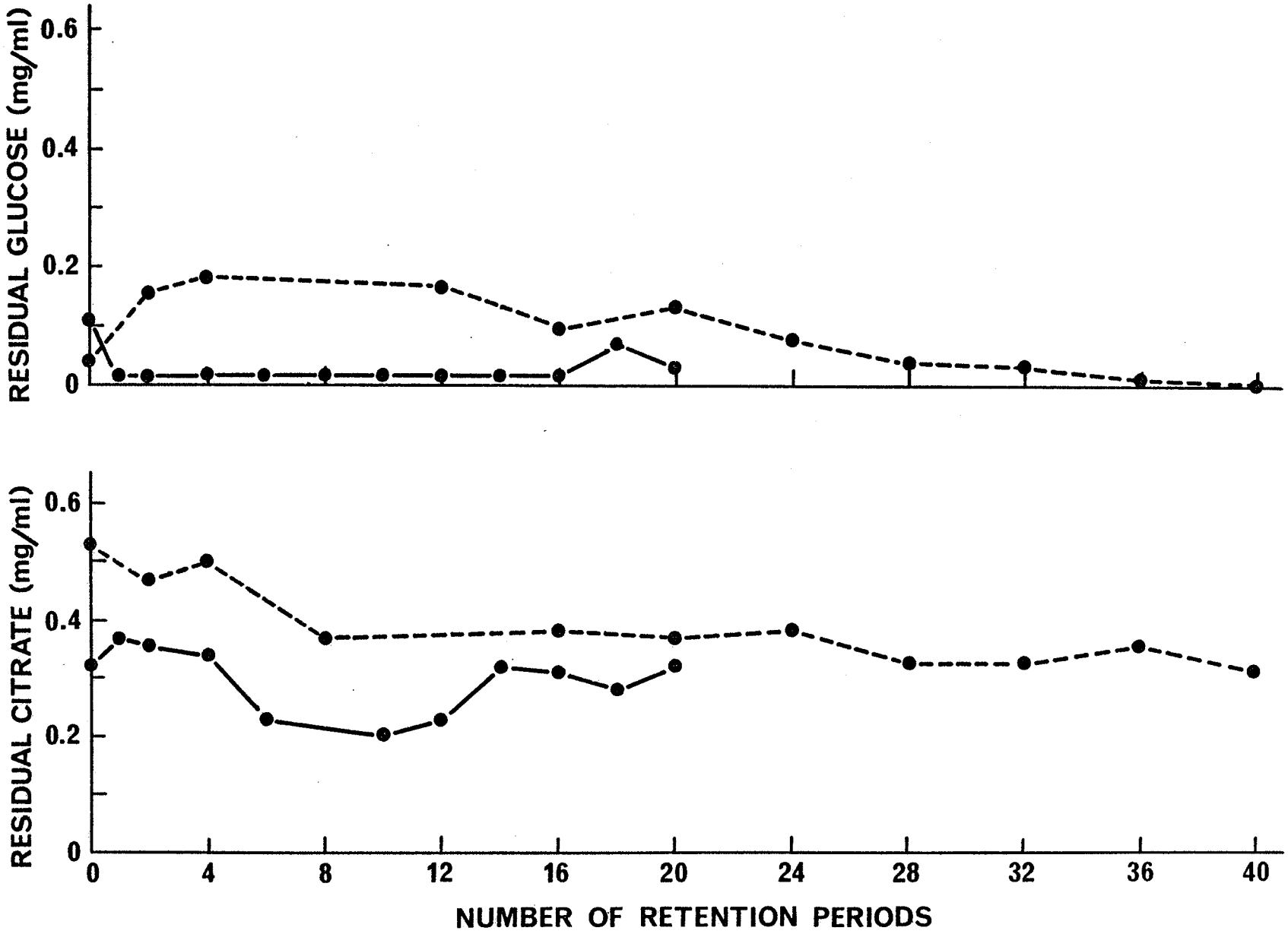

RESIDUAL GLUCOSE (mg/ml)
0.6
0.4
0.2
0
RESIDUAL CITRATE (mg/ml)
0.6
0.4
0.2
0
0
4
8
12
16
20
24
28
32
36
40
NUMBER OF RETENTION PERIODS

Fig. 12 Concentration of residual acetate in the medium C mixed culture.

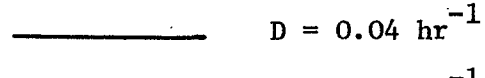
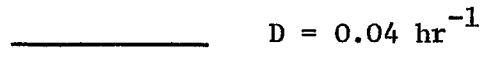


Fig. 13 Concentration of residual lactate in the medium C mixed culture.



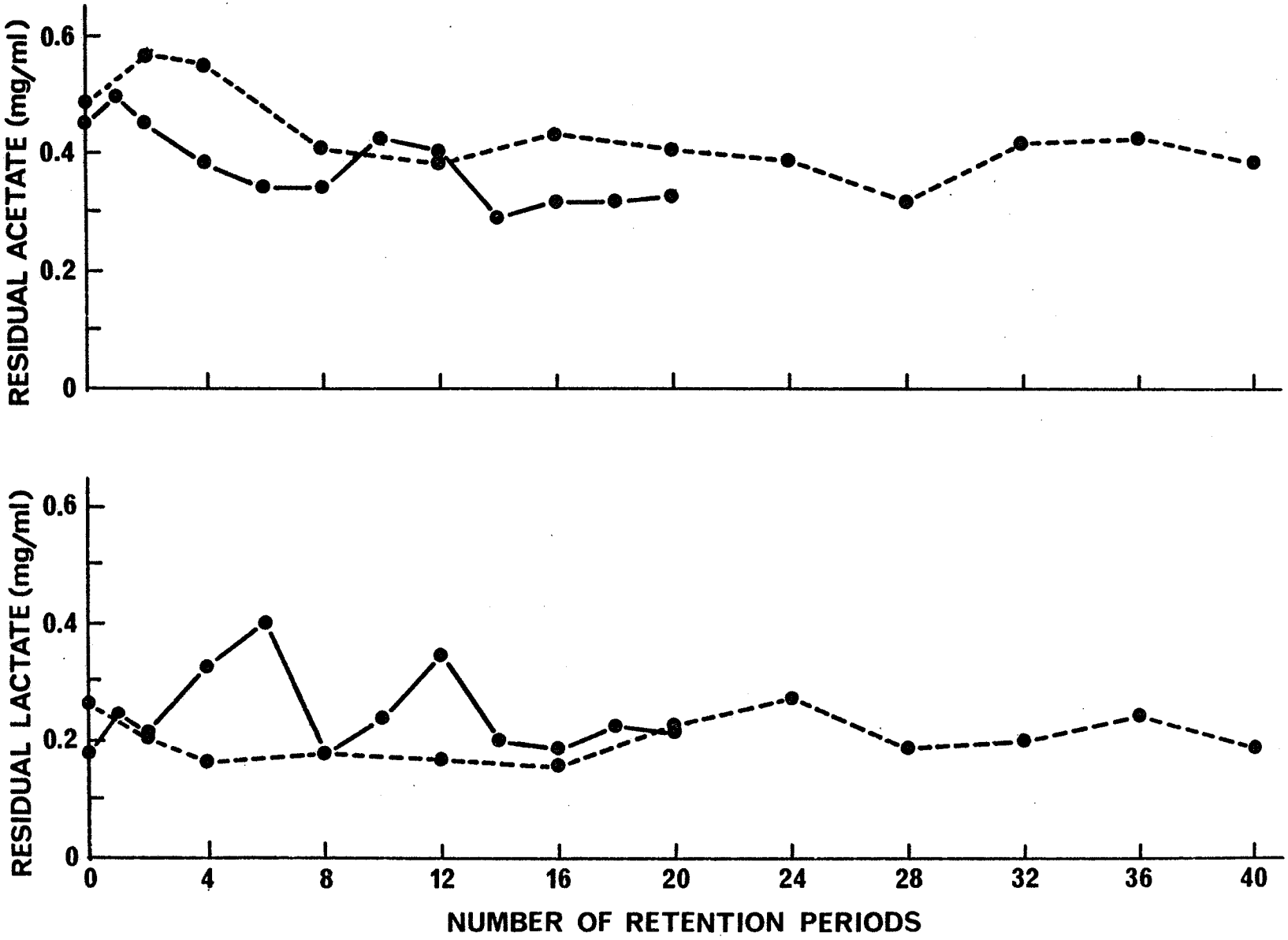
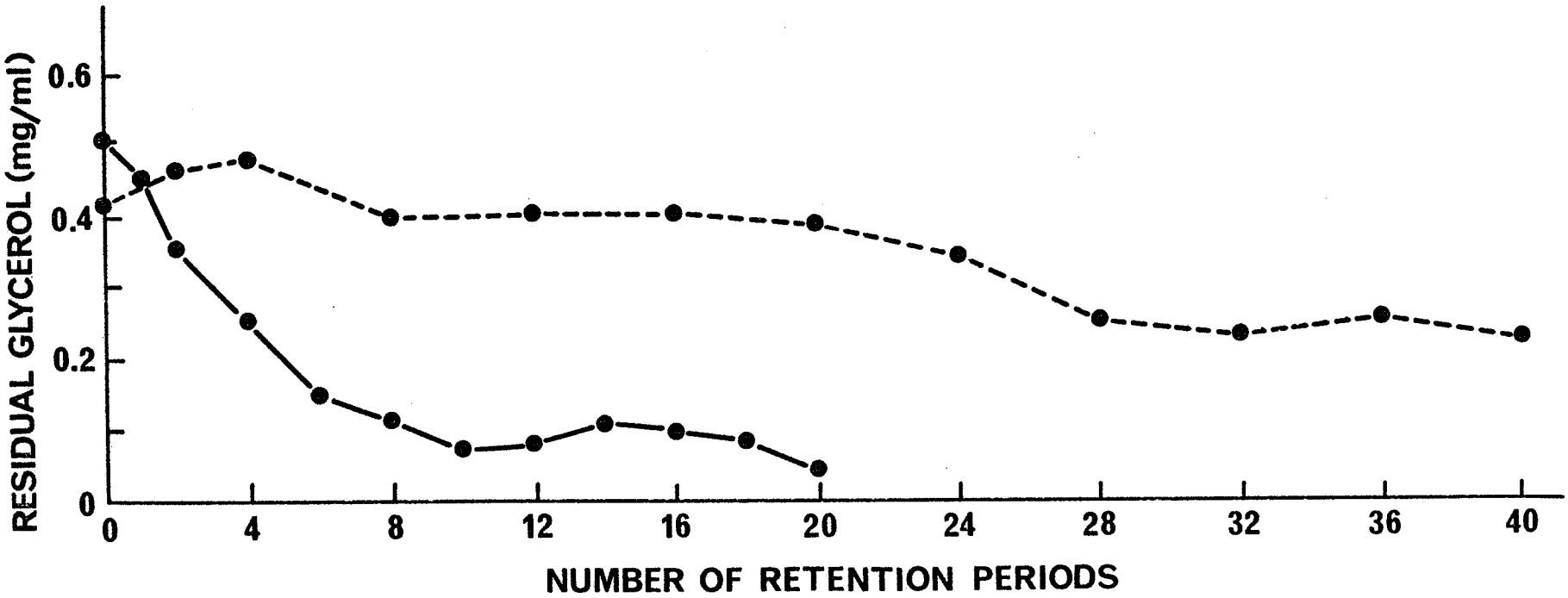

RESIDUAL ACETATE (mg/ml)
0.6
0.4
0.2
0
RESIDUAL LACTATE (mg/ml)
0.6
0.4
0.2
0
0
4
8
12
16
20
24
28
32
36
40
NUMBER OF RETENTION PERIODS

Fig. 14 Concentration of residual glycerol in the medium C mixed culture.

———————— D = 0.04 hr^{-1}

---------- D = 0.08 hr^{-1}


RESIDUAL GLYCEROL (mg/ml)
0.6
0.4
0.2
0
0
4
8
12
16
20
24
28
32
36
40
NUMBER OF RETENTION PERIODS

glucose level increased from 40 to 180 µg/ml. The remaining 36 retention periods, however, saw a continued decline in the residual glucose in the culture to a point at R40 where it could no longer be detected in the spent medium.

Citrate levels, Fig. 11, remained relatively high in both mixed cultures when compared to glucose. Growing at a dilution rate of 0.08 hr^{-1} the culture utilized very little of the citrate from the start of dilution to R4, although by R8 the citrate concentration had decreased to a steady-state level of approximately 0.35 mg/ml. Reducing the dilution rate to 0.04 hr^{-1} allowed a greater percentage of the citrate to be metabolized by the culture, however, a uniform concentration was not established during the 20 retention periods.

The uptake of acetate by the mixed populations, shown in Fig. 12, appeared to be rather limited. In batch growth neither mixed culture utilized a significant amount of the acetate present in the medium. When the dilution rate was started at 0.08 hr^{-1} approximately 20% of the acetate was being consumed by the eighth retention period and a reasonably steady-state level of 0.4 mg/ml was maintained throughout R40. The mixed culture growing at the slower rate utilized a greater percentage of the available acetate, but again fluctuations in substrate concentration beyond R4 were observed.

Lactate utilization by the mixed cultures was not only insignificant at both dilution rates but was also less uniform over the operational period of the chemostats, Fig. 13. At D = 0.08 hr^{-1} the bacteria reduced the lactate concentration in the medium to a constant 0.17 mg/ml

from R4 through R16. In the samples analyzed from R20 to R24 however the residual lactate increased to a high of 0.28 mg/ml before declining to a stable level of 0.20 mg/ml. The data generated at D = 0.04 hr^{-1} were very erratic and indicated pronounced changes in the residual lactate concentration. Samples analyzed at R4, R6 and R12 contained lactate in excess of the 0.25 mg/ml originally present in the medium.

In Fig. 14, the utilization of glycerol by both mixed cultures is shown. Although glycerol did not appear to be a favoured substrate under the conditions of batch growth, its uptake following the start of dilution was unquestioned. The mixed culture grown at a rate of 0.08 hr^{-1} consumed a constant 20% of the medium glycerol from R8 through R20. In the ensuing samples the residual glycerol further declined to establish a new steady-state level from R28 through R40 during which time approximately 50% of the substrate was now removed from the medium. The lower dilution rate resulted in a very different pattern of glycerol uptake. Commencing with the start of dilution, the glycerol concentration in the culture decreased continually until the tenth retention period, at which point it attained a steady-state. Between R10 and R20 the residual glycerol amounted to only 0.08 mg/ml and is equivalent to 84% of the substrate consistently utilized by the mixed culture.

DISCUSSION

DISCUSSION

The three nutritionally-balanced growth media used in this study are known to support a wide range of soil isolates based upon standard enumeration methods (48, 57, 93). Our results show that these media are also capable of maintaining the simultaneous growth of several soil organisms in a chemostat. The exact number of different organisms arising from the soil inoculum did vary with the formulation of the particular medium. Peptonized milk medium and synthetic sewage medium consistently supported the growth of more types of bacteria than were found in medium C. During one experiment, eight different isolates were obtained from peptonized milk, seven from synthetic sewage and only four from medium C. The principal difference between the former two types of media and the latter is the supply of growth factors to the organisms. The ability of undefined growth factors to permit the growth of a greater diversity of soil organisms has been known for some time (94). Sundman and Carlberg (93) reported that the number of soil isolates capable of growth on medium C could be substantially increased if the medium was supplemented with a number of growth factors.

It is also noteworthy that the very dilute peptonized milk medium was able to accommodate the nutrient requirements of at least as many if not more, soil organisms than the more concentrated medium formulation of synthetic sewage. This has also been observed by Larkin (57), who found that peptonized milk with actidione was superior to either Trypticase soy agar or soil extract agar for the enumeration of soil

bacteria. Therefore a maximum number of soil organisms can be obtained from a dilute medium formulation containing growth factors. Peptonized milk medium at a concentration of 0.1% fulfils these requirements, is easy to prepare and will allow the coexistence of a reasonably wide variety of different soil organisms in a chemostat. The only difficulty with the peptonized milk medium was the significant amount of attached growth that developed on the stirrer and walls of the reactor. This limited the maximum length of chemostat operation to only three weeks.

Undoubtedly the three media when used in a continuous culture situation, cannot support the range of bacteria normally isolated directly from the soil. This is because soil is a very heterogeneous nutrient environment especially in relation to the rigid conditions imposed by the chemostat. As discussed by Taylor and Williams (95), regardless of the number of species present in the original inoculum, the number of bacterial types existing in the chemostat at steady-state cannot exceed the number of growth-limiting components in the reactor. It is this statement, supported by the theory of the chemostat, that restricts a broad spectrum medium like peptonized milk from supporting more than seven or eight different organisms.

Nevertheless the diversity of our mixed cultures compared favourably to a number of natural environments other than soil. Prakasam (83) isolated six different organisms from activated sludge while Cherry (17) reported an average of eight to ten isolates from the aquatic environments of ponds and streams. The diversity of organisms

is significantly less and therefore the heterogeneous populations generated in the chemostat can serve as a reasonable model for such natural environments. The restrictions on such a comparison have been discussed by Jannasch (51).

The biochemical differentiation of the colonies isolated from peptonized milk and synthetic sewage cultures (Tables 1 & 2) ensured the isolates selected on the basis of colony characteristics were truly distinctive from each other. The salient feature of the isolates was the predominance of Gram-negative motile rods. Furthermore, these Gram-negative organisms were almost exclusively non-enterics according to the oxidase and High-Leifson tests. The sole enteric isolated from the mixed cultures was a Klebsiella sp. (Table 1,#7). A Bacillus sp. obtained from the peptonized milk culture proved to be the only Gram-positive organism present (Table 1,#1&2). Other isolates identified were Pseudomonas aeruginosa (Table 2, #5), based upon the characteristic diffusible pyocyanine pigment and a Xanthamonas sp. (Table 2, #4), based upon the colony morphology and weak oxidase reaction.

The natural advantage of Gram-negative non-enteric organisms in continuous cultures has been observed by other authors. Meers & Tempest (70) invariably found the Gram-negative Enterobacter aerogenes and Pseudomonas fluorescens displacing the Gram-positive Bacillus and Staphylococcus species from the mixed culture over a wide range of dilution rates. The competitive elimination of enterics from the mixed culture chemostat has been described by Jannasch (50). He found low substrate levels and low dilution rates precluded the establishment of enterics when in competition with marine organisms. Considering the

operational characteristics of our chemostats which included source of inoculum, low dilution rates (0.04 hr^{-1}), relatively low temperature ($20^{o}C$), and in the case of peptonized milk medium, low nutrient level (0.1%), it is not suprising that enteric organisms were unable to become established in the mixed cultures.

The differences in the composition of the three mixed cultures were not only reflected in the number and nature of the colony types isolated, but also in their ability to metabolize the casamino acids substrate measured in terms of respiratory rates (Fig. 2). Casamino acids were used as substrate for all manometric experiments since Bashucky (2) has found it to be an acceptable means of standardizing the active biomass of sewage lagoon bacteria. Therefore oxygen uptake in response to casamino acids was considered a useful physiological parameter to assess mixed cultures. On a dry weight basis the peptonized milk and synthetic sewage mixed cultures had approximately equivalent rates of oxygen consumption, whereas medium C-grown cells displayed a much lower rate of respiration. Since all three cultures possessed about 10^{9} cells/ml, the low respiration rates of the medium-C culture are likely a reflection of their previous growth history.

In the examination of steady-state in the mixed cultures, respiration rate was used as a measure of physiological stability with viable cell count and dry weight as the criteria for biomass stability. It was found that the nature of the parameter used as well as the mixed culture tested influenced the conclusions reached in regard to the achievement of steady-state (Figs. 3, 4 & 5). Biomass stability measured by dry weight appeared to be the least sensitive parameter and displayed a

minimum amount of fluctuation in all three mixed cultures. Total biological solids has been used extensively as a method to verify steady-state in heterogeneous populations (30, 31, 97). On this basis the data imply that the biomass had stablized by the fifth or sixth retention period.

Only in the case of the mixed culture grown on peptonized milk medium does the second biomass parameter, viable cell count, support the preceding conclusion. By the fourth retention period the peptonized milk culture attained a maximum cell density which remained constant for the last 200 hours of chemostat operation (Fig. 3). The medium C mixed culture also reached a stable viable biomass during the last 200 hours of dilution, however, 12 turnover times were required before this steady-state was established (Fig. 5). The lengthy stablilization of the medium C culture was again evident at the dilution rate of 0.08 hr^{-1} where 20 retention periods elapsed before a stable viable count was achieved (Fig. 6). Therefore the data reported for total viable cell count suggest the five or six turnover periods necessary to establish a steady-state biomass, as indicated by dry weight, may be an underestimation. In fact as many as 20 retention periods were sometimes required before the viable biomass attained stability.

Mateles and Chian (68) have also cautioned that the acclimatization process of mixed cultures to continuous dilution may require 10 to 25 residence times compared to the five or six allowed for pure cultures to reach steady-state. In addition, the stability of a heterogeneous

population at steady-state may never compare with the results obtained with pure cultures. Mateles and Chian (68) observed 10-20% fluctuations in the cell density after 15 residence periods. Hendricks (40) found similar results throughout the 400 hours of dilution at a rate of 0.012 hr^{-1}. During steady-state in peptonized milk and medium C mixed cultures the total viable biomass displayed a variance of less than eight percent.

In contrast to the reasonable stability in biomass of the mixed cultures, the respiration rates of the populations continued to fluctuate over the steady-state interval. One possible explanation for the lack of correlation between the total viable count and the oxygen uptake of the mixed culture may be due to changes in the relative species composition in the population not detected when measuring the total biomass. Blok (5) has employed respirometric data as an indication of the active biomass in activated sludge and found it to be a more precise method of evaluating the condition of the population when compared to the procedures of total biological solids, effluent COD, etc. Bashucky (2) has also used oxygen uptake to evaluate mixed populations of sewage lagoon bacteria. It was concluded that good correlation existed between the respiratory rates calculated from manometric data and the active biomass present in the suspension.

The extent of individual species fluctuations occurring in the mixed culture was tested using the simple population generated in medium C (Tables 3 and 4). In both experiments the same three organisms were present with isolate B dominating the mixed batch culture, but giving

way to isolate A once the dilution had been initiated. During the operation of the chemostat the ratio of the two organisms exhibited noticeable changes not always reflected by the total viable count. For example, at D = 0.04 hr^{-1} (Table 3) the total viable count at R14 was 8.5 x 10^8 cells/ml and the ratio of organism A to organism B was five to one. Two retention periods later, at R16, the total count remained essentially the same, 8.2 x 10^8 cells/ml, yet organism A was now in excess of organism B by a margin of 13 to one. Similarly at D = 0.08 hr^{-1} (Table 4), viable counts for R20 and R28 remained constant at 8.2 x 10^8 and 8.0 x 10^8 cells/ml respectively, while the ratio of A to B decreased from eight down to four. It is apparent, therefore, that the species composition of the culture can oscillate without expressing an effect on the total biomass. This fact together with the knowledge that the respiration rate of the casamino acids substrate can differ for each species in the mixed culture may contribute to greater fluctuations in the $Q(O_2)$ of the population than is observed in its total biomass at steady-state.

The number of individual species comprising a heterogeneous culture is not usually examined in most reports. Cassel et al. (16) based individual fluctuations of constituent organisms on the quantitative analysis of the pigment composition in the reactor. Two types of instability were observed; (a) one or two pigments predominated continuously, but changed in concentration from day to day; and (b) several pigments appeared and disappeared in irregular fashion. The

data for medium C corresponded more closely to the first situation since organisms A and B dominated the two mixed cultures continuously over the 500 hours of operation, yet their numbers constantly fluctuated with respect to one another. Instances of the second type of instability were also recognized. At sporadic intervals during the experiment the presence of a yellow-pigmented colony was noted which attained a density of 10^5 - 10^6 cells/ml before declining to an extent where it was no longer evident in the culture.

In evaluating steady-state in continuous culture it is necessary to consider the effect of attached bacterial growth. Irrespective of the intensity of stirring in the reactor, organisms begin to adhere to the walls of the growth vessel, the magnetic stirrer and the effluent tube. The degree of bacterial adherence to solid surfaces is known to be a function of a number of variables including culture concentration, temperature, nature of the growth medium, type of solid surface involved and the bacterial species present (28, 58, 69). After three weeks of continuous operation the attached growth was most severe in the peptonized milk culture and least noticeable in medium C. Since all the cultures were grown under identical physical conditions and were found to contain approximately 10^9 cells/ml, the copious amounts of capsular material associated with the peptonized milk culture which facilitated attached growth, must be solely a consequence of the medium composition. It is interesting that the presence of casein in the peptonized milk medium has been shown to enhance the attachment of bacteria to solid surfaces (69).

Herbert et al. (41) were the first to mention the effect of wall growth on the theoretical predictions of steady-state. They stated that the sloughing off of attached growth provides a continual inoculum to the suspended culture. Hendricks (40) found the numbers of his heterotrophic population oscillated with a periodicity of approximately 100 hours and attributed this interval to the time-dependent build-up and sloughing off of wall growth into the suspended culture. Larsen & Dimmick (58) indicated a significant fraction of the bacteria in suspension are aggregated in minute flocs arising from detached wall growth. When the culture was exposed to brief sonication prior to enumeration, the plate count values consistently increased suggesting the release of individual organisms from the floc. In a two-membered mixed culture of Chromobacterium lividum and a Pseudomonas sp., Maigetter and Pfister (65), noted that the accumulation of wall growth was exclusively due to the adherence of C. lividium whereas the Pseudomonas sp. showed no such tendency.

Therefore the inevitable build-up of attached growth may be due to only certain species present in the mixed culture which would be a selective advantage to those organisms existing in the chemostat. The periodic release of these organisms from the wall of the reactor would increase their presence in the mixed culture resulting in fluctuations in the parameters used to evaluate steady-state. In fact the attainment of a reasonable steady-state in a mixed culture may be partially

dependent on the time required for the release of attached growth to occur at a relatively constant rate.

Due to the fact that a great deal of mixed culture experimentation is conducted with reference to waste water treatment efficiency, steady-state has been routinely measured in terms of the consistency of effluent composition, usually referred to as 'effluent COD' (16, 34). The defined formulation of medium C permitted the quantitative analysis of individual carbon sources to determine if there was selectivity of carbon uptake by the mixed culture and whether the effluent substrate levels remained relatively constant.

The results indicated the organisms present in the mixed culture displayed a preference for glucose and glycerol as substrates. Citrate was moderately consumed while little of the acetate and lactate was removed from the medium (Figs. 10-14). The repression of oxidation of less favoured substrates in continuous culture has been shown to occur in systems operated at low dilution rates where carbon is a non-limiting component of the medium (18, 33, 39). In this situation the presence of readily degraded carbon sources such as glucose or glycerol will be metabolized by the bacteria in excess of their immediate biosynthetic requirements. Consequently the levels of metabolic intermediates within the cell will be high. This type of environment is therefore condusive to catabolite repression of less favoured substrates such as acetate and lactate.

Not only was the nature of the carbon source a factor in its removal by the organisms, but also the rate of its dilution had a noticeable affect. The residual substrate level was normally lower in the chemostat operating at the slower dilution rate. This observation is consistent with the theory of the chemostat which predicts at lower dilution rates the removal of the growth-limiting component(s) in the medium will become more efficient (41).

In general the residual carbon in the effluent was slightly more consistent in the chemostat operated at 0.08 hr^{-1} than at 0.04 hr^{-1}. The analyses of the residual substrate levels again indicate the need for extended periods of acclimatization of the mixed culture to continuous dilution. At D = 0.08 hr^{-1} the glucose and glycerol concentrations continued to decline until approximately the thirtieth retention period. At the lower dilution rate stability in the concentrations of citrate, acetate and lactate was not attained throughout the entire experiment.

In conclusion it has been shown that of the three media evaluated the dilute peptonized milk medium was simple to prepare and yet fulfilled the nutrient requirements of a reasonably heterogeneous culture growing in a chemostat environment. The assessment of steady-state with respect to mixed cultures has indicated that total biomass measured as viable cells is superior to the method of dry weight. However, even the total viable cell count will not reflect oscillations of the individual species comprising the mixed culture which were found to occur. These internal changes in species composition were exhibited in the physiological

parameter of respiratory rates. The $Q(O_2)$ values for the mixed cultures generally fluctuated to a greater extent than either biomass parameter. Therefore oxygen uptake represented the most sensitive method used to evaluate stability in the mixed cultures. It has also been firmly established from the data obtained that stability of mixed cultures will not adhere to the theoretical principles of steady-state normally accepted in pure culture studies. However, even with less stringent standards for steady-state it is the conclusion of this work that 10 to 15 residence periods are required to attain the maximum stability in the mixed culture rather than the five or six turnovers routinely allowed for pure culture.

PART II THE STARVATION SURVIVAL OF MIXED BACTERIAL CULTURES

INTRODUCTION

INTRODUCTION

The study of endogenous metabolism and survival of bacteria in a nutrient-free environment has been previously restricted to pure cultures usually generated from a batch culture system (22, 23). Work in this laboratory, however, routinely involves the preparation of resting cell suspensions from mixed bacterial populations which are subsequently used for varying periods of time (2, 54). The results from pure culture studies indicate that a number of factors can affect the length of survival of a resting cell suspension which may range from one or two days to several months (81, 27). It was therefore considered necessary to evaluate the survival properties of mixed bacterial populations generated from a soil-inoculated chemostat. In a study of this type it is advantageous to grow the organisms in continuous culture since variations in survival characteristics due to growth phase and culture conditions are reduced to a minimum (81). In this manner a comparison between the starvation survival capability of the mixed culture and the medium formulation used for its generation can be made. In addition, several parameters were tested to assess the survival of the resting cell suspensions in order to determine a reliable method for estimating the active biomass remaining.

HISTORICAL

HISTORICAL

Endogenous metabolism may be defined as the sum of all metabolic activities occurring within an organism when suspended in the absence of utilizable extracellular substrates. Subjecting bacteria to a non-nutrient environment leads to a progressive loss of cell biomass caused by the imbalance in total anabolic and catabolic reactions within the cell. Historically, endogenous metabolism has had only nuisance value to the investigator, since this metabolism intruded upon quantitative estimations of the oxidation of external substrates principally measured by manometric procedures. To resolve this technical problem of endogenous metabolism it was considered a convenient solution to simply subtract the endogenous respiration measured in the absence of substrate from the total oxygen consumed by the cells when supplied with an exogenous substrate. Such a cursory treatment of endogenous metabolism does not, however, satisfy an understanding of the intracellular reserves of the organism, their variation with environmental conditions, and their biosynthesis and subsequent dissimilation under the influence of nutrient deficiency.

Since 1960 reports have appeared specifically studying the endogenous metabolism of microorganisms, including two early reviews by Dawes and Ribbons (22, 23). Postgate (82) has also presented a review on the subject while the New York Academy of Sciences published a complete symposium (55) encompassing a wide variety of topics on endogenous metabolism in prokaryotic and eukaryotic cells.

The function of endogenous metabolism as envisaged by Dawes & Ribbons (22) is: (a) to serve as an energy source permitting the cell to continue such energy-demanding processes as osmotic regulation, maintenance of intracellular pH, motility, and the turnover of cellular protein and nucleic acid; (b) to provide carbon skeletons from which degraded cellular components may be resynthesized; (c) to perform certain special functions in the cell, such as supplying a source of reducing power to chemoautotrophic and photoautotrophic bacteria. In assessing the survival potential of a resting cell population, three criteria must be considered: (a) the nature of the bacterial species being studied; (b) the physical and chemical environment to which the organisms are exposed during starvation; (c) the previous nutritional history of the organism.

This latter point was thought to be of considerable importance, since bacterial storage compounds, namely glycogen and poly-β-hydroxybutyrate (PHB), have traditionally been regarded as important substrates in endogenous metabolism. An early report by Ingram (47), correlated the decline in endogenous respiration rate of Bacillus cereus with the utilization of cellular lipid. Macrae and Wilkinson (64) also investigated the role of PHB in the endogenous metabolism of B. cereus and Bacillus megaterium. In environments where nitrogen became growth-limiting, the cells continued to accumulate cellular carbon in the form of PHB. Upon exposure to a nutrient-free environment the cells degraded these intracellular reserves as a carbon and energy

source which presumably retarded the rate of cell death and autolysis since these events occurred more rapidly in PHB-poor cells. Pseudomonas saccharophila suspended in a nitrogen-free, glucose or acetate medium stored from 60-80% of its assimilated carbon as PHB which was readily metabolized in the absence of an exogenous carbon source (25).

The multiple functions of intracellular reserves were demonstrated by Doudoroff & Stanier (25) in the organism Rhodospirillum rubrum, a facultative photoautotroph. Incubation of washed suspensions of R. rubrum with ^{14}C-acetate resulted in the deposition of 70% of the assimilated ^{14}C into PHB. When the cells were transferred to phototrophic conditions (i.e. presence of light in a CO_2:N_2 atmosphere) in a medium free of organic carbon but containing an added nitrogen source, more than 90% of the PHB was consumed with much of the ^{14}C being redistributed to other cellular components. The rate of PHB degradation was reduced if exogenous acetate was supplied to the medium, but this did not occur if succinate was added. Studied more closely by Stanier et al. (88), it was found that R. rubrum photoassimilated succinate into glycogen reserves. Therefore the accumulation of either PHB or glycogen by this organism depended upon the chemical structure of the exogenous substrate supplied. In addition they showed that break-down of PHB or glycogen during starvation was dependent upon the presence of CO_2. It was concluded that during a time of nitrogen availability the cellular reserves of PHB or glycogen can be used as a supply of carbon skeletons for the synthesis of nitrogenous compounds as well as

a source of reducing power for further CO_2 assimilation.

The observation that the nature of the carbon source available dictates the type of storage compound formed in R. rubrum emphasizes the importance of the growth environment in controlling the accumulation of cellular reserves. This is particularly relevant in situations where exogenous carbon is not in excess in the medium and hence cannot be sequestered as storage material. The question raised by Dawes & Ribbons (24) concerns the relative importance of such storage compounds as PHB and glycogen in preventing the degradation of more basal cell constituents and thereby preserving the viability of the population.

Dawes and co-workers have documented a number of experiments involving effect of growth conditions on the endogenous metabolism and viability of Escherichia coli (21, 24, 85) and Sarcina lutea (9, 85). Grown on a glucose-ammonium salts medium, E. coli contained 2-3% cellular glycogen. Once suspended in phosphate buffer at 37°C, the cells rapidly depleted these reserves within one hour and with it a concomitant drop in the endogenous respiration rate. Following the degradation of cellular glycogen, the cells began to release measureable quantities of ammonia into the buffer. RNA was also undergoing net degradation at this time as detected by its cellular decline and the release of u.v.-absorbing compounds. In contrast, E. coli grown on tryptone-ammonium salts medium failed to accumulate glycogen, and when exposed to starvation, the cells immediately exhibited a loss of ammonia to the suspending

medium. Interestingly in neither situation did the cellular protein nor the free amino acid pool become depleted during the period of ammonium excretion. Although protein degradation has not been excluded, later data have suggested that following the depletion of glycogen, oxygen consumption can in part be accounted for by the oxidation of ribose produced in the degradation of RNA (23). The investigators concluded that the presence of glycogen in E. coli prevented the net degradation of more important nitrogenous compounds in the cell. Strange (92) has stated that glycogen-rich E. coli and Enterobacter aerogenes are capable of surviving extended periods of starvation. In this respect the rapid utilization of available glycogen by E. coli is difficult to comprehend. Dawes and Ribbons (23) have on the other hand suggested that in E. coli glycogen serves only as a reserve of carbon skeletons and not an endogenous source of energy. Experimental support for this concept has been reported by Holme & Palmstierna (45), who showed that when supplied with a source of nitrogen the ^{14}C label, originally present only in the cellular glycogen, quickly becomes redistributed into cellular protein.

The deposition and fate of glycogen in E. coli cannot be compared to the results obtained with S. lutea (9, 85). When harvested from a glucose-peptone medium these cells contained approximately 12% respirable carbohydrate. The utilization of this reserve however did not prevent the concurrent oxidation of the amino acid pool nor the extracellular release of ammonia. Furthermore the amino acid pool was not replenished from cellular protein and the depletion of

this pool closely correlated the fall in the endogenous $Q(O_2)$ value. These results indicate that the free amino acid pool is the primary endogenous reserve for S. lutea and the presence of additional reserve carbohydrate does not retard the degradation of nitrogenous compounds nor prolong viability. In fact S. lutea rich in polysaccharide was reported to survive less well than those organisms which were deficient in the polymer (9).

Campbell et al., (14, 103) have studied the endogenous metabolism of Pseudomonas aeruginosa and have concluded that cellular protein serves as the primary reserve material of the cell. During the initial two hours of starvation in phosphate buffer at 30°C, the total consumption of oxygen was accounted for by the decline in cell protein which was completely oxidized to CO_2 and NH_3. Extending the starvation period beyond two hours initiated the degradation of ribosomal RNA with the concomitant release of 260 nm- absorbing material into the buffer. Neither DNA nor carbohydrate was degraded.

Thomas and Batt (98) also found cellular RNA and protein to function as endogenous substrates in Streptococcus lactis, an organism that did not accumulate polysaccharide or PHB. Unlike P. aeruginosa, however, RNA was favoured over protein as a substrate since the amount of protein lost from the cell over 30 hours of starvation was less than five percent. The degradation of RNA was not accompanied by complete intracellular utilization as was indicated by both 260 nm-absorbing and orcinol-reactive material being detected in the supernatant.

Strange et al. (89) have stated that the viability of E. aerogenes can be extended in a buffered suspension given the cells are rich in glycogen. Grown to stationary phase on glucose-tryptone medium most of the organism's 15-20% carbohydrate was in the form of glycogen. When deprived of nutrients the stored glycogen was efficiently consumed while little RNA was lost from these cells even after the glycogen had become depleted. In comparison this same organism grown in a carbon-limiting chemically-defined medium contained no glycogen and consequently relied on RNA oxidation for survival. In a subsequent report Strange et al. (90) found the degradation of ribosomes supplied both the RNA and the protein that was metabolized by these cells. They therefore agreed with Mendelstam & Halvorson (67) who concluded that in E. coli the degradation of ribosomes supply most of the free amino acids and all of the ribonucleotides passing through the free internal pools during starvation. The above reports give firm evidence that, in the absence of net protein synthesis and where conventional storage compounds have not been synthesized, ribosomes become an expendable yet highly utilizable endogenous substrate (36, 67, 90).

Although reports differ widely on the relative importance of polysaccharide, lipid, protein and RNA to bacterial survival, it is generally considered that regardless of the organism studied or its nutritional environment DNA is a stable cell constituent and is not utilized during starvation (14, 44, 90).

The type of nutrient environment used to generate the cells however is not the only criterion establishing the intracellular chemical

composition of the organism. The growth phase of the population exposed to nutrient deprivation has also been shown to affect survival since the chemical makeup of a cell differs in exponential and stationary phase populations. Parnas & Cohen (77) explain that the cellular synthesis of storage material should only occur when the organism is approaching a period of starvation. Therefore in batch growth where carbon is limiting, only during the transition to stationary phase when carbon concentration has fallen to a low level will the organism begin to accumulate storage material. If these reserves are important to an individual organism's survival, then suspensions prepared from stationary phase cells will survive longer than cells taken from an exponential phase of growth. The authors cited as supporting evidence the work by Holme & Palmstierna (45) who showed that the degree of glycogen accumulation in E. coli was most predominant towards the end of stationary phase. Strange et al. (89) have reported that suspensions of E. aerogenes harvested from the late stationary phase survived better than cells harvested from early stationary or mid-log phase cultures. Their observations were in agreement with an earlier study made by Harrison (38). In Pseudomonas aeruginosa, an organism which does not accumulate storage materials, resting cells prepared at any stage of growth survived equally well (14). Only in the experiments involving Arthrobacter crystallopoietes have resting cell suspensions prepared from exponentially-growing cells been demonstrated to have superior survival compared to stationary phase cells (27).

The survival period of an organism in a resting cell population will undoubtedly be controlled by the physical and chemical conditions of the starvation environment. Postgate & Hunter (81) have carried out an extensive investigation on the relationship between a number of environmental factors and the survival of E. aerogenes. They found that the optimum temperature (20°C) and pH (6.5) of starvation were quite different from the optimum growth condition of the organism. The need for suspending the cells in a buffered medium was also demonstrated. Druilhet and Sobek (26) found that phosphate buffer was superior to either Tris or EMTA buffers in suspensions of Salmonella enteritidis. Postgate and Hunter (81) added a chelating agent to their buffer which removed minute quantities of Cu^{+2} present in the distilled water that was toxic to the cell suspension. On the other hand, the presence of Mg^{+2} or Ca^{+2} extended the viability of the population. Holden (44) showed that the addition of Mg^{+2} prevented the degradation and leakage of nucleic acids from Lactobacillus arabinosus. He proposed that polybasic anions such as $PO_4^{\equiv}$ initiated RNA degradation by the removal of protective cations. The stabilizing effect of added Mg^{+2} on RNA has also been demonstrated with E. coli (6), S. lactis (98) and S. lutea (9).

Harrison (38) was the first to observe that the population density of the suspension affected cell survival. Suspensions with a cell density of 10^5 cells/ml did not survive as well as suspensions prepared at a density of 10^8-10^9 cells/ml. Similar population effects have been found to occur with E. aerogenes (81) but not with S. lutea (9).

Harrison (38) believes that at low cell densities, intracellular material lost to the buffer through early cell lysis or excretion becomes diluted to such an extent that the remaining cells have no opportunity to recover this material before most of the population dies. At higher densities the extracellular materials may become more concentrated in the buffer permitting the extended viability of the remaining cells due to their reassimilation of a portion of these constituents. He found that by continually transferring the resting cells to fresh buffer during their starvation, it was possible to significantly shorten their expected viability when compared to cells retained in the same buffer throughout the experiment. This indicates the ability of the bacteria to modify the composition of the buffer making it more suitable for survival of the population and presumably this modification occurs more rapidly at high cell densities than at low cell densities.

In their investigation of the starvation survival of E. aerogenes, Strange et al. (89) noted that the addition of glucose (0.45% w/v) to the resting cell suspension markedly accelerated the death rate of the population. The presence of substrate resulted in a 50% loss of viability in the resting cells within four hours of incubation when normally no cell death occurred during this time if suspended in buffer alone. Postgate and Hunter (81) also demonstrated substrate-accelerated death in E. aerogenes starved in the presence of glycerol. Loss of viability paralleled the decline in $Q(O_2)$ and glycerol dehydrogenase activity in the cell suspension. Substrate-accelerated

death has also been reported in S. lactis (98) and in an Arthrobacter species (27). In resting cell suspensions of Arthrobacter the exogenous addition of glucose did not facilitate measureable RNA or protein synthesis. Calcott andPostgate (12, 13) have pursued the study of substrate-accelerated death in resting cells and have revealed the following points. While glycerol-grown cells suspended in buffer exhibited an accelerated death rate when starved in the presence of glycerol or pyruvate, this is not observed if the cells are stored in the presence of glucose. The reverse is also true, that is, cells grown on glucose will have an enhanced death rate when starved in the presence of glucose or pyruvate but not when exposed to glycerol. Furthermore, the addition of Mg^{+2} or cyclic-AMP to the substrate-enriched buffer will prevent the expression of all substrate-accelerated death. At this time the authors believed that the addition of substrate did not actually kill the cells but caused a severe repression of substrate catabolism which was not overcome when transferred to the recovery medium. Both Mg^{+2} and c-AMP are involved in relieving this suppression. The effect of exogenous substrate on resting cells is extremely important and should be considered when doing experiments of extended duration such as found in manometric studies.

While the potential for survival of a given resting cell suspension can be manipulated by the previous growth environment of the organism and by the conditions under which starvation occurs, the capability of the population to survive nutrient deprivation is largely a function of the nature of the bacterial species under investigation. Vegetative bacterial cells of different species, exposed to a similar non-nutrient

environment, will withstand the rigors of starvation for periods of time ranging from a few hours to several weeks (27). In resting cell suspensions of S. lactis starved at 30°C in phosphate buffer, 50% of the viability was lost within the first 30 hours of starvation and by the second day the suspension was completely non-viable (98). Another Gram-positive organism, S. lutea, also exhibited a rapid loss of viability with only 25% of the population remaining viable after three days at 37°C in phosphate buffer (9). Cell suspensions of E. coli, starved under identical conditions, lost 50% of their viable cells after 36 hours and 75% within two days (24). The survival capability of E. aerogenes was maximized when glycogen-rich cells were generated in tryptone-glucose medium. These cells retained 95% viability for 65 hours and 50% viability after four days (89). S. enteritidis starved in phosphate buffer at 37°C exhibits the best survival ability so far reported for organisms belonging to the enteric group. Druilhet and Sobek (26) have found complete viability in their resting cell suspensions of S. enteritidis after 60 hours of starvation which declined to approximately 50% after six days. Therefore, in organisms whose endogenous metabolism has been described earlier, the half-life of the population generally falls within one to six days of starvation.

Lamanna and Mallette (56) have stated that evolution should select for starvation-resistant organisms, however this may only be necessary for bacteria normally subjected to the stress of starvation. This statement has received supporting evidence from studies made of the survival of certain soil and marine bacteria. A number of authors have reported the long-term survival of Arthrobacter species (7, 27, 63, 107) while Robertson and Batt (86) have found similar survival properties

for the actinomycete Nocardia corallina. Both of these organisms are considered as members of the autochthonous soil microflora described by Winogradsky (105). This group of microorganisms composes a relatively stable population in the soil and are generally characterized by slow growth rates and slow death rates allowing them to maintain viability during periodic deficiencies of nutrient. In phosphate-buffered suspensions of Arthrobacter globiformis and A. crystallopoietes the 50% survival time for the populations was 42 days and 100 days respectively (27, 63) while Nocardia corallina suspensions displayed a 50% viability after 20 days of starvation (86).

The most characteristic feature of these organisms is their ability to rapidly step down the rates of endogenous respiration by over 90% within the first two days of starvation. Ensign (27) showed that the endogenous $Q(O_2)$ value for A. crystallopoietes fell from 9.0 to 0.1 by the second day and remained constant at this rate for the remainder of the experiment. As this basal level of endogenous metabolism it was calculated that only 0.03% of the cellular carbon was being consumed per hour, yet this was still sufficient to meet the requirements of the organism for at least 30 days since no loss of viability could be detected in the cell suspension during this time.

The pattern of endogenous metabolism of organisms capable of long-term survival is analogous to the description outlined for bacteria of short survival times. Zevenhuizen (107) found that a glycogen-like polymer accumulated by an Arthrobacter species was the sole endogenous substrate for the first five days of starvation. When starvation is extended and the carbon-containing reserves become depleted, intracellular protein and RNA will serve as substrate with

the products of their degradation, ammonia and u.v.-absorbing bases, being released into the medium (7).

Novitsky and Morita (76) have recently shown that similar characteristics of long-term survival occur in a marine vibrio. The psychrophilic nature of the organism required growth and starvation to be performed at 5°C. At this temperature the half-life of the resting cell suspension was 40-50 days and within the first week of starvation the endogenous respiration rate had been attenuated to less than one percent of its original value.

The minimum energy requirements needed to sustain the essential processes for continued cell survival has been termed the "energy of maintenance" (66). The ability of a cell to fulfil its maintenance energy demands during prolonged periods of starvation may be a reflection of the intracellular economy of the organism rather than the presence of additional reserves of endogenous substrates. Boylen and Ensign (7) have stated that the maintenance energy for A. crystallopoietes is comparable to those values reported for E. coli and E. aerogenes (79) and for S. lactis (99) which are equivalent to the utilization of 0.045 - 0.09 mg glucose mg^{-1} of cells hr^{-1}. However the endogenous respiration rates for organisms capable of long-term survival are five to ten times lower than the rates measured for E. coli or S. lactis.

The literature suggests that organisms isolated from the marine environment or comprising the autochthonous microflora of the soil have evolved stringent control over their rates of endogenous metabolism. This control permits the consumption of endogenous substrates at a

rate supplying the minimum energy required to maintain the starved cell in a viable state.

With the knowledge that such a wide variety of factors may influence the starvation survival of a bacterial cell, it was of interest to establish the survival properties of resting cell suspensions prepared from a heterogenous microbial population.

MATERIALS AND METHODS

MATERIALS AND METHODS

Preparation of Resting Cell Suspensions

Mixed bacterial cultures obtained from the effluent of the three different chemostats were used in the preparation of resting cell suspensions. Between 900 and 1000 ml of effluent culture was collected over a 24 hour period in a clean Erlenmeyer vacuum flask packed completely in ice. Following their collection the cells were harvested and washed as described on page 26. The washed cells were resuspended in 50 ml of 0.1M potassium phosphate buffer, pH 7.0. The final suspension contained 4-5 mg/ml dry wt of cells.

Experimental Outline

The prepared resting cell suspensions were used to study changes in the survival, respiratory rates, and biochemical composition of the bacteria under starvation conditions. Experiments, performed at three incubation temperatures, 4^{o}, 20^{o}, and $37^{o}C$, were generally conducted over a one week period. During this time the cell suspensions were continually shaken and at selected intervals samples were removed and assayed for survival measured as viable count or cellular ATP, cellular respiratory rate, and biochemical composition including nucleic acids (both RNA and DNA), carbohydrate and protein. Leakage of cellular components was also measured by following the release of nucleic acids, carbohydrate and protein into the suspending buffer.

Methods of Analysis

i) Cell survival: Viability was determined by the ability of the cells to multiply and form visible colonies on an agar surface. The viable cell count was performed using the method on page 27. Trypticase-soy agar plates were used for all determinations of viable cell count since it has been shown that organisms placed under environmental stress will lose their ability to divide when plated on minimal media much more rapidly than if a nutritionally-rich non-selective medium is used (4, 38).

ii) Cellular adenosine triphosphate: Cellular adenosine-5'-triphosphate (ATP) was assayed using the firefly luciferin-liciferase method after suitable extraction (62, 78). In vitro light production by firefly lantern extract is directly proportional to the amount of ATP when in the presence of luciferin, the enzyme luciferase, magnesium ions and oxygen.

The extraction of ATP from the cells was accomplished in the following procedure. One ml of cell suspension was pipetted directly into a 25-ml volumetric flask containing 15 - 20 ml of boiling 20mM Tris buffer (pH 7.75). Extraction continued for five minutes after which time the volumetric flask was cooled and made up to volume with sterile Tris buffer. The extract was frozen at -15^{o}C for later analysis.

The ATP content of the extract was determined using Worthington firefly extract (Worthington Biochemical Corp., Freehold, N.J.) in conjunction with an ATP photometer (Model 2000 JRB Inc., La Jolla, Calif.). Each vial of luciferin-luciferase reagent was rehydrated

with 5.0 ml distilled water and allowed to stand at room temperature for 30 minutes prior to use. The photometer was zeroed and each sample or standard was counted over a 45 second time interval where the light emitted under a decay curve was measured. The samples were corrected for background activity which could be reduced by performing the assay in the absence of fluorescent light. The quantity of ATP present was determined by referring to a standard curve.

The stock solution of ATP was prepared by dissolving 119.3 mg ATP (disodium salt; from Sigma Chemical Co., St. Louis, Mo.) in 100 ml of sterile 20mM Tris buffer (pH 7.75) containing 29.2 mg EDTA and 120 mg $MgSO_4.7H_2O$. The resulting solution contained 1 mg/ml ATP and was used in the preparation of standards from 0 to 1.0 µg ATP.

Nucleic acids: The resting cells were analyzed for both RNA and DNA content following perchloric acid extraction (42). A volume of cells having a dry weight of 20-60 mg was spun down at 13,000 rpm (20,200 x g) for 15 minutes in a model RC2-B Sorvall Superspeed centrifuge (Ivan Sorvall Inc., Norfolk, Conn.). The supernatant was decanted and stored for the anlaysis of extracellular nucleic acids, carbohydrate and protein. The cell pellet was resuspended in 5 ml of 0.25N $HClO_4$ and allowed to stand for 30 minutes in an ice-water bath with occasional mixing. The suspension was centrifuged and the acid-soluble material in the supernatant discarded. The cell pellet was then suspended in 4 ml of 0.5N $HClO_4$ and placed in a 70°C water bath for 15 min with periodic stirring, followed by centrifugation. The extraction was

repeated twice more with 3-ml volumes of 0.5N $HClO_4$, each for 15 minutes. The three extracts were pooled and made up to 10 ml if necessary with 0.5N $HClO_4$.

RNA was analyzed by the orcinol method of Herbert et al. (42) using D-ribose as the standard. DNA was determined by the diphenylamine procedure of Burton (10) with calf thymus DNA serving as the standard. Both the ribose and the DNA were products of Sigma Chemical Co. (St. Louis, Mo.).

Carbohydrate: Carbohydrate was measured as total hexose by the anthrone method (42).

Protein: Extraction of the cellular protein was accomplished by adding 0.5 ml of an appropriately diluted suspension into 0.5 ml of 1.0N NaOH and placed in a boiling water bath for 5 minutes. Protein was determined according to the procedure of Lowry et al. (61). Bovine serum albumin (Sigma Chem. Co., St. Louis, Mo.) was used as the standard.

Absorbance measurements for the colorimetric determinations of RNA, DNA, carbohydrate and protein were made on a Beckman DB spectrophotometer (Beckman Inst. Inc., Fullerton, Calif.).

All chemicals used in the chemical analysis were standard reagent grade.

RESULTS

RESULTS

Standard Curves for Quantitative Analysis

The standard curves for the interpretation of the data to follow are found in Figs. 15 and 16. The standard curve for ATP analysis is shown in Fig. 15. Standard curves for RNA, DNA, carbohydrate and protein are shown in Fig. 16.

Survival of Resting Cells

Mixed bacterial populations generated by continuous culture methods were used to prepare resting cell suspensions in 0.1M phosphate buffer. It has been shown in Part I of the present study that each of the three medium types used in the chemostat produced a unique population of soil organisms. This made it of interest to examine the effect of medium formulation on the survival characteristics of the bacteria in a non-nutrient environment. The starvation experiments were performed at the original growth temperature of 20°C as well as at 37° and 4°C.

The initial experiment was a simple viability assay, to establish the percentage of the original population that remained viable over six days of starvation at each of the three incubation temperatures. The results obtained for medium C-grown and synthetic sewage-grown cells are shown in Fig. 17. It was immediately apparent that both the incubation temperature and the previous growth conditions of the

Fig. 15 Standard curve for the quantitative analysis of ATP.

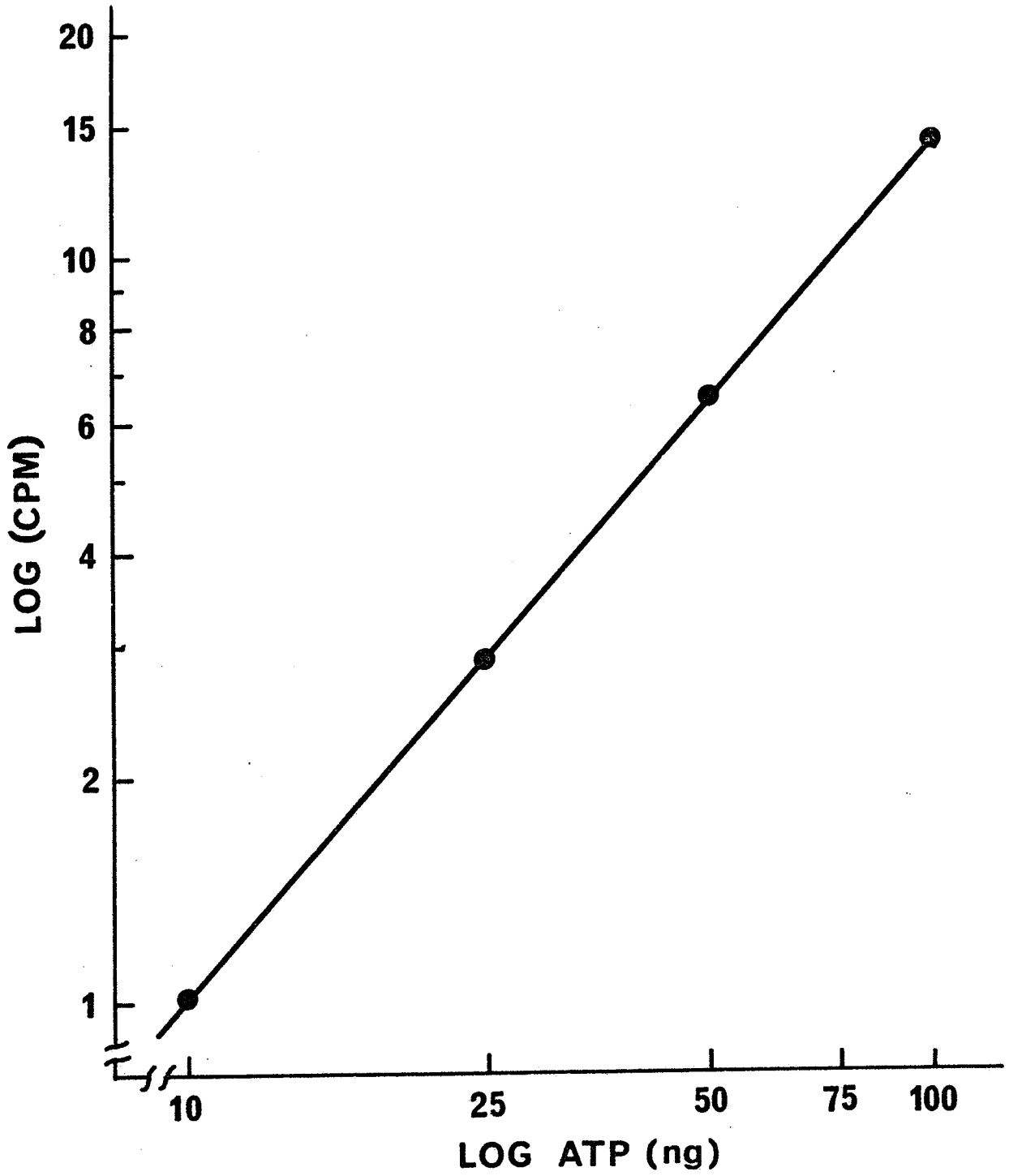

20
15
10
8
6
4
2
1
LOG (CPM)
10
25
50
75
100
LOG ATP (ng)

Fig. 16 Standard curves for the quantitative analysis of

— — — — DNA @ 600 nm

—.—.—.— RNA @ 672 nm

———————— Carbohydrate @ 625 nm

---------- Protein @ 750 nm

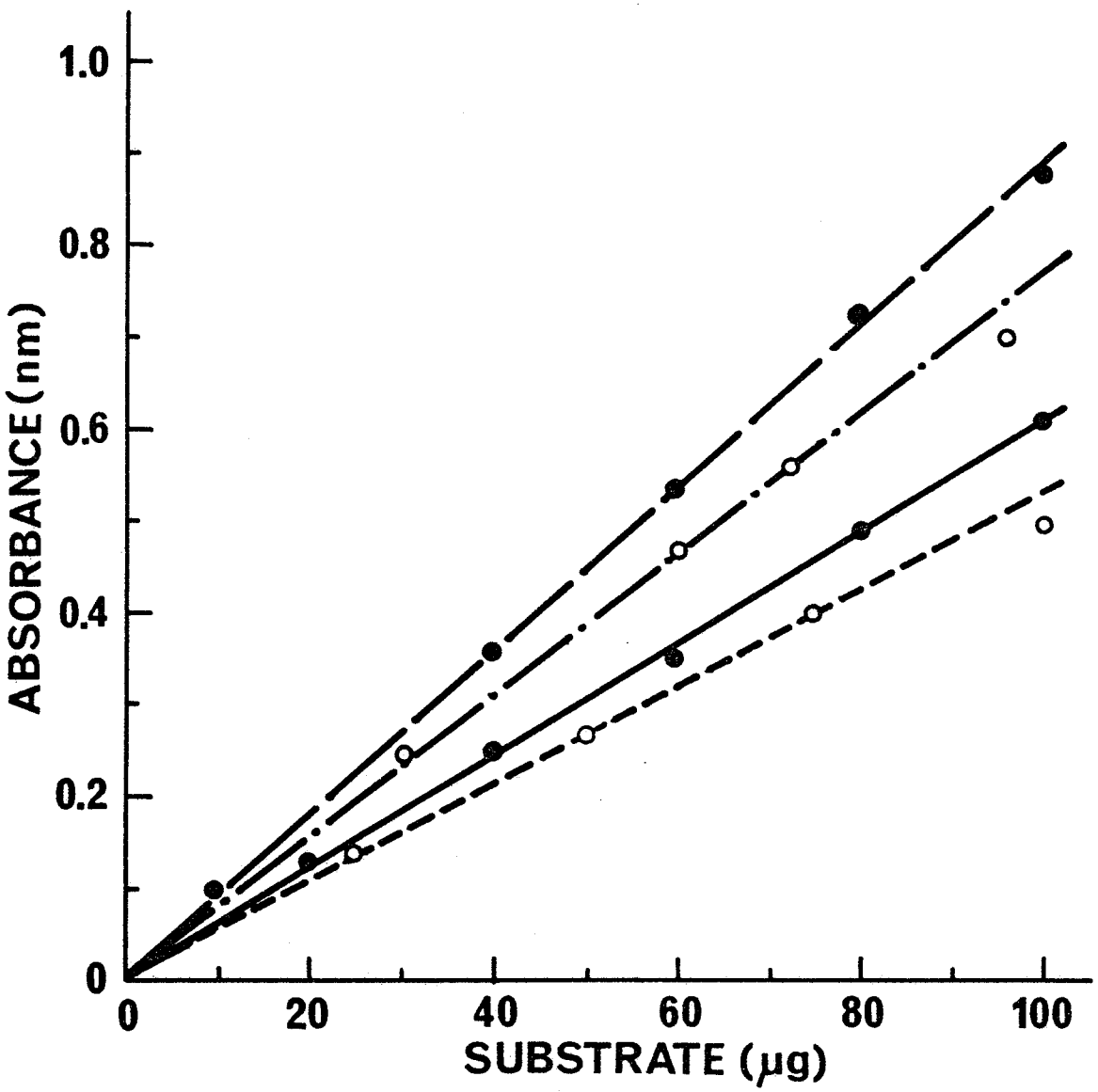
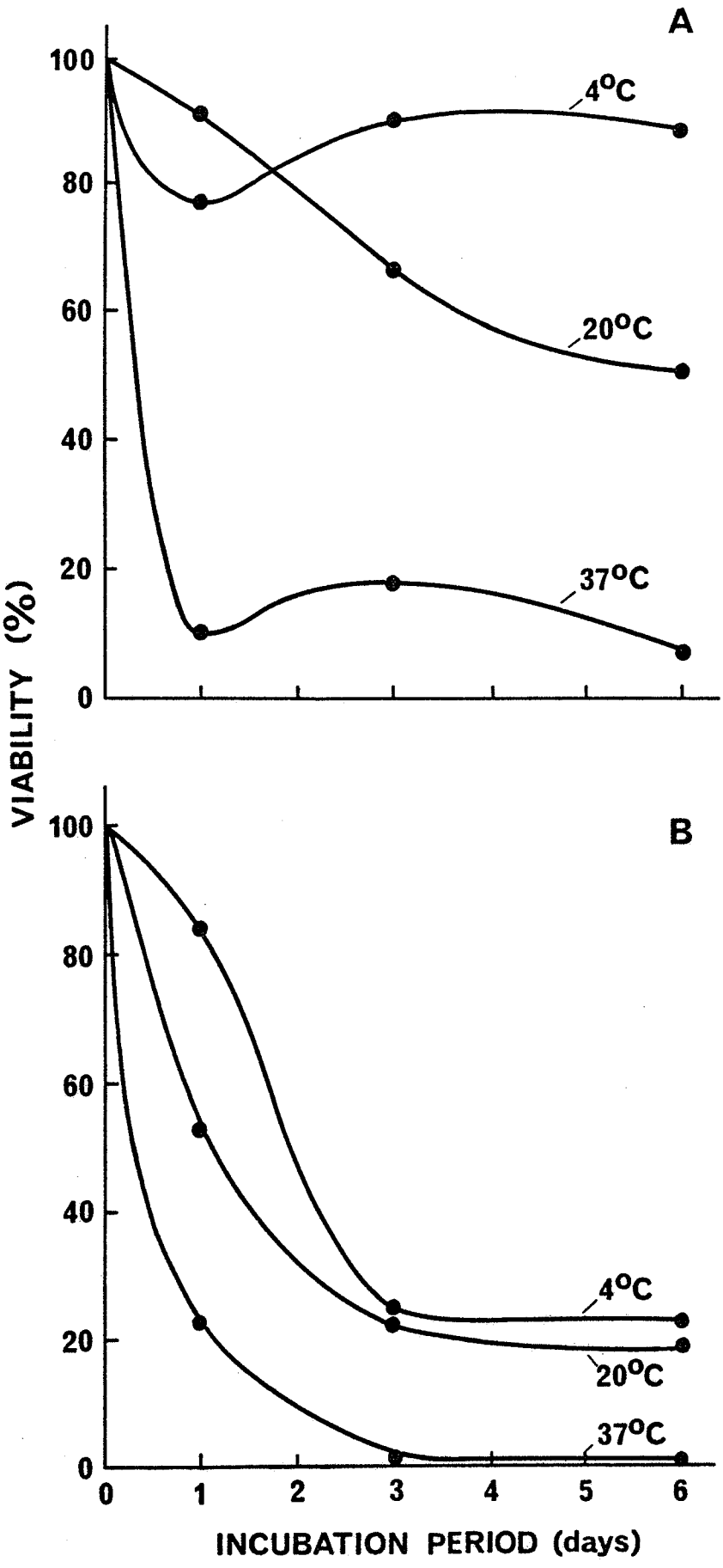

1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE (nm)
0
20
40
60
80
100
SUBSTRATE (µg)

Fig. 17 Survival of resting cell suspensions grown on

(a) A: Medium C

(b) B: Synthetic sewage


A
100
80
60
40
20
0
4°C
20°C
37°C
VIABILITY (%)
B
100
80
60
40
20
0
4°C
20°C
37°C
0
1
2
3
4
5
6
INCUBATION PERIOD (days)

population significantly affected the mortality rate of the cells. In Fig. 17a, the lowering of the temperature during starvation markedly prolonged the life of the medium C-grown cells. Whereas only 7% of the original population remained viable following six days of storage at 37°C, approximately 50% of the cells were viable if the incubation temperature was lowered to 20°C and this percentage could be further increased to 90% provided the cell suspension was refrigerated at 4°C.

The apparent protective effect of low temperature on cell survival of medium C-grown cells was not demonstrated in resting cell suspensions prepared from synthetic sewage medium (Fig. 17b). In these cell suspensions 77% of the viability was lost within three days of storage at 20°C and lowering the temperature to 4°C still permitted the loss of 75% of the cell population. At 37°C only 1-2% of theinitial population had colony-forming ability by the third day of starvation. The significance of such contrasting responses to starvation as measured by viable count will be discussed.

The standard plating procedure was found to be unreliable when enumerating viable cells in suspensions prepared from peptonized milk medium. The often erratic counts were believed to be the result of copious amounts of extracellular slime associated with the organisms which at the high cell densities used facilitated the clumping of cells, thereby under-estimating the initial viable count. However, after hours of continual agitation, better dispersion of the cells occurred as indicated by a substantial increase in the cell number. In

this situation cell death was masked by the liberation of viable cells from the tiny flocs.

In order to obtain a more accurate evaluation of the peptonized milk-grown cells, it was decided to measure the cellular ATP content of the suspension as an indicator of viable biomass. ATP content of the freshly-prepared suspension was considered to represent 100%. It can be seen from Fig. 18 that some increase in cellular ATP did occur at $4^{o}C$ and $37^{o}C$, but not at $20^{o}C$. At $37^{o}C$ this elevated level of ATP lasted only one day after which the µg ATP/mg dry wt of suspension falls exponentially, resulting in only 15% of the initial content present at Day six. The cells starved in buffer at $4^{o}C$ also showed a rise in ATP content which persisted during the first three days of incubation. Loss of viability was slow at this temperature; after six days of starvation, 90% of the population remained viable. At $20^{o}C$ cell death was linear through the initial four days and tended to level off after this point; approximately 50% of the cells were viable at Day six.

Physiological Stability of Resting Cells

The oxidation of intracellular materials during starvation was measured by the endogenous respiration rate of each of the prepared suspensions. The results expressed as µl O_2/hr/mg dry wt and referred to as the $Q(O_2)$ value are shown in Fig. 19. Cells generated from the peptonized milk medium (Fig. 19a) displayed an initial $Q(O_2)$ of nine to 12. Storage of the suspension at $4^{o}C$ reduced this rate by half during

Fig. 18 Cellular ATP of starved resting cell suspensions grown on peptonized milk medium.

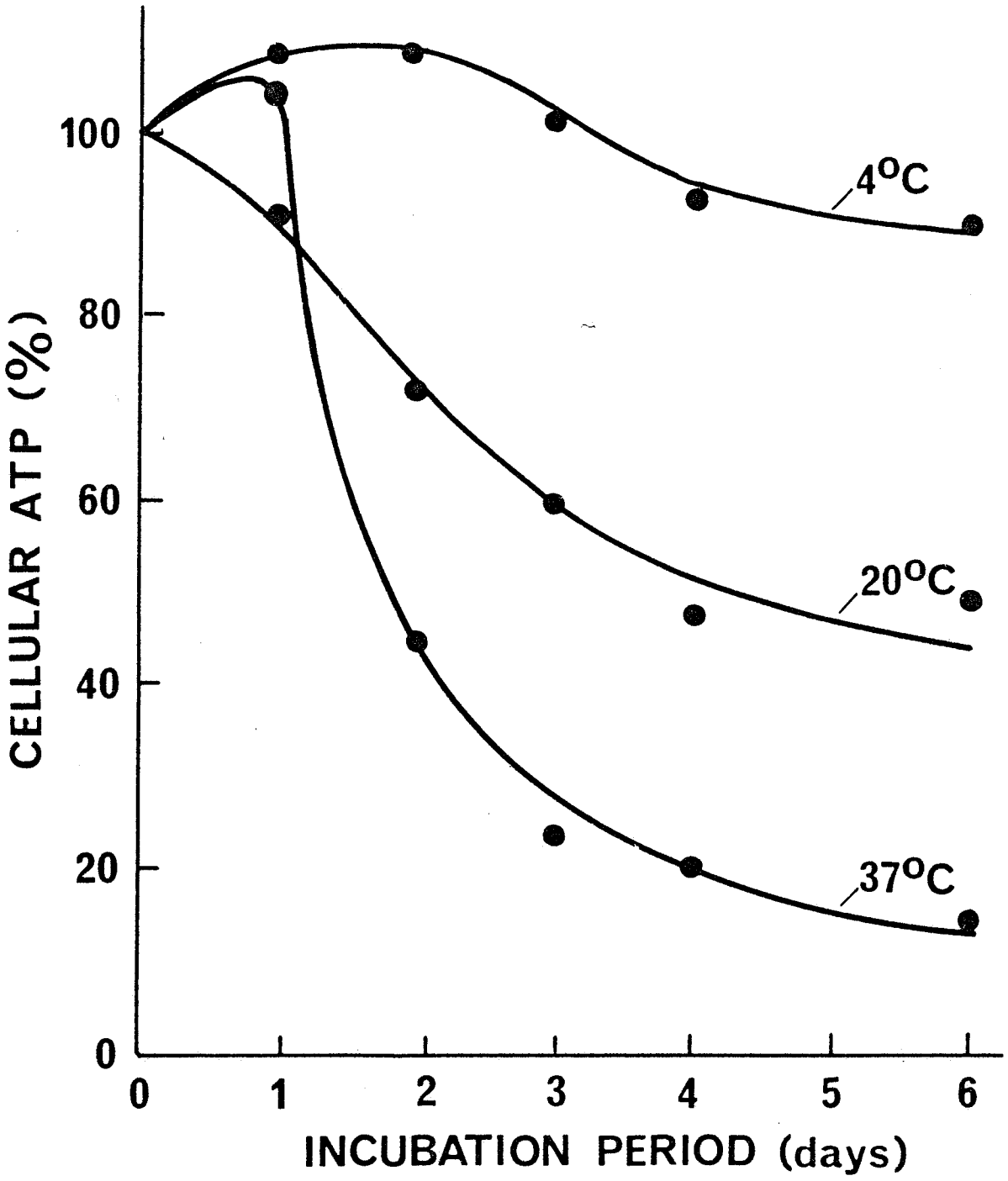
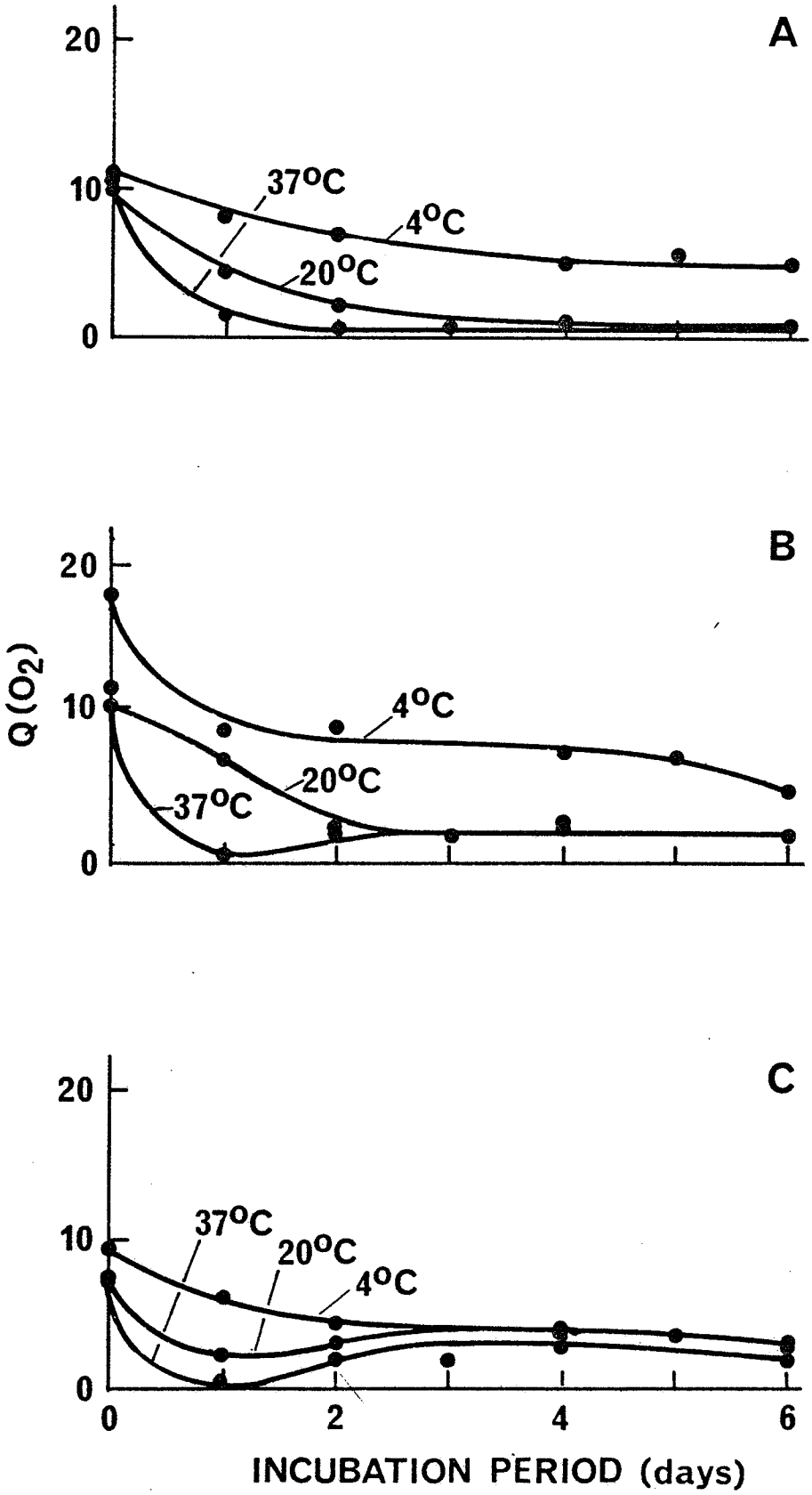

CELLULAR ATP (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
INCUBATION PERIOD (days)
4°C
20°C
37°C

Fig. 19 Endogenous respiration of resting cell suspensions grown on

(a) A: Peptonized milk medium

(b) B: Medium C

(c) C: Synthetic sewage medium


A
20
10
0
37°C
4°C
20°C
B
20
10
0
Q(O2)
4°C
20°C
37°C
C
20
10
0
37°C
20°C
4°C
0
2
4
6
INCUBATION PERIOD (days)

the first four days and throughout the sixth day the respiration rate remained at five µl O_2/hr/mg dry wt. Increasing the incubation temperature to 20°C resulted in the $Q(O_2)$ of the suspension decreasing to almost immeasurable values by the fourth day. At 37°C the endogenous respiration rate of the cells was identical to that recorded at 20°C, except that a $Q(O_2)$ value of 1-2 was reached within the first two days of starvation.

The endogenous respiration of cells grown on medium C (Fig. 19b) was quite similar to the results obtained for the peptonized milk-grown cultures. Both the 20°C-and the 37°C-starved cells exhibited the same $Q(O_2)$ value of 2.5 by the second day of storage. In addition, the cells starved at 4°C again retained a higher $Q(O_2)$ throughout the experiment. It should be noted however that the initial $Q(O_2)$ value for this suspension was slightly higher; 16.5 compared to the 10-12 value normally associated with these cells.

The most characteristic observation of the resting cells prepared from synthetic sewage medium (Fig. 19c) was the absence of a high endogenous respiration rate at 4°C. By the fourth day of starvation both the 4° and 37°C cell suspensions had an endogenous $Q(O_2)$ of three to four. It was also noted that synthetic sewage-grown cells had the lowest initial $Q(O_2)$ value, usually between seven and nine, of all three mixed cultures.

The resting cell suspensions were also tested for their ability to respond to exogenous susbtrate after starvation. The respiration rates of the cells when challenged with a mixed substrate (vitamin-free

casamino acids) are shown in Fig. 20. The oxidative capability of the cells from all three sources was surprisingly high during a six day period, provided the suspension was incubated at 4° or 20°C, but was entirely lost if the cells were kept at 37°C. The $Q(O_2)$ values of the peptonized milk-grown cells (Fig. 20a) decreased linearly during starvation at 4° and 20°C, with the rate of decline in activity being 1.8% and 4.3% per day respectively. At this rate the suspension stored at 4°C retained 70% of its initial activity after 16 days of starvation, while cells stored at room temperature retained 50% of their original $Q(O_2)$ after 11 days. When the storage temperature was increased to 37°C the exogenous respiration rate declined exponentially and by the end of the sixth day these cells were no longer capable of responding to the external substrate.

Fig. 20b also suggested that the decline in amino acid oxidation activity of medium C-grown cells was linear at 4° and 20°C, but exponential at 37°C. The exogenous $Q(O_2)$ was 90% of its initial value after 16 days at 4°C and 74% of its initial value after 12 days starvation at 20°C.

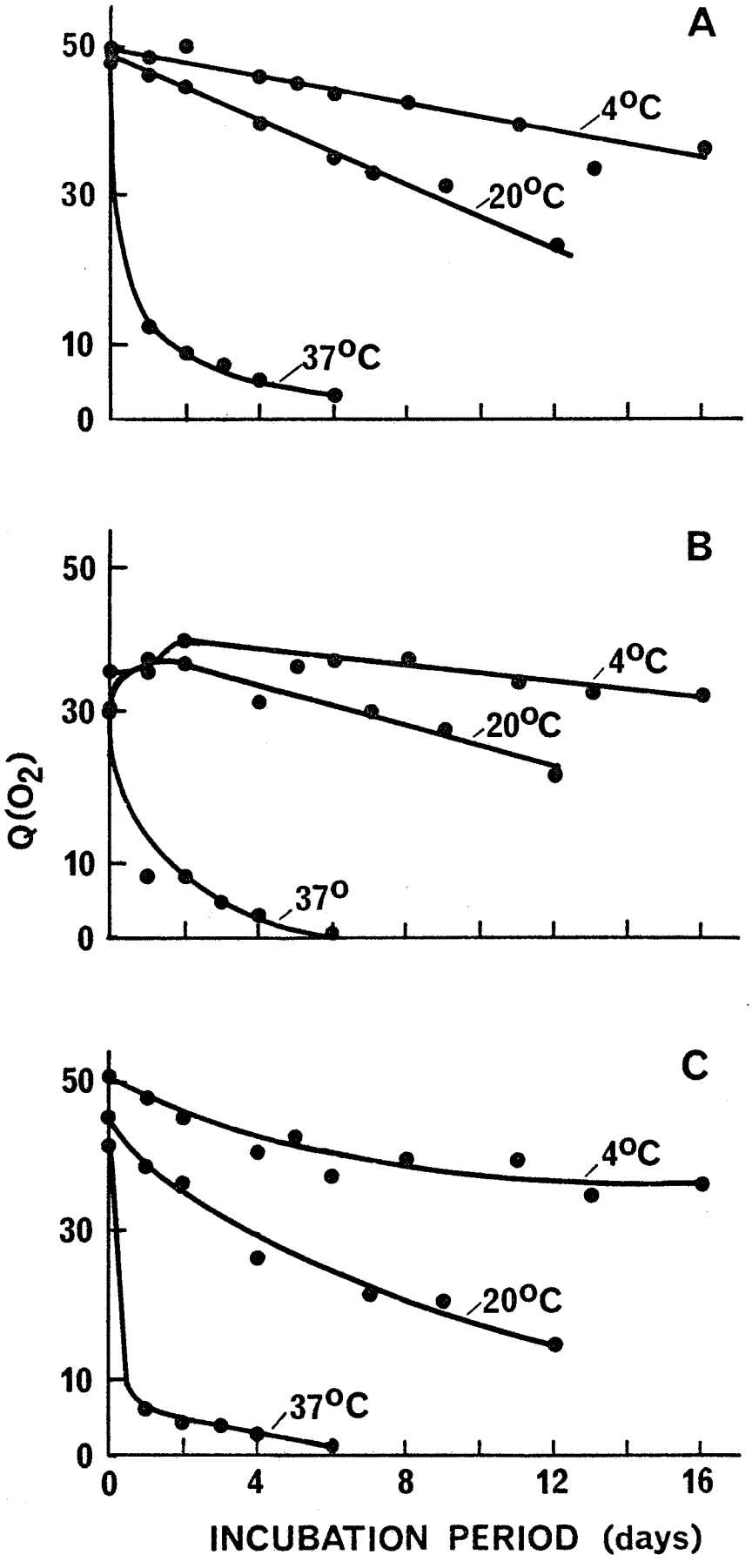
With cells harvested from the synthetic sewage medium (Fig. 20c) the loss of respiratory activity was more curvilinear at the temperatures of 4° and 20°C. The data showed that 27% of the original $Q(O_2)$ was lost at 4°C after 16 days and 67% of the activity was lost after 12 days at 20°C. These values are significantly larger than were noted in the previous figures.

Fig. 20 Exogenous respiration of resting cell suspensions grown on

(a) A: Peptonized milk medium

(b) B: Medium C

(c) C: Synthetic sewage medium


A
50
30
10
0
4°C
20°C
37°C
B
50
30
10
0
Q(O2)
4°C
20°C
37°
C
50
30
10
0
4°C
20°C
37°C
0
4
8
12
16
INCUBATION PERIOD (days)

Biochemical Stability of Resting Cells

It was decided to determine if any changes in the biochemical composition of the cells during starvation could be detected. Such chemical analyses may aid in the understanding of the events leading to a loss of metabolic activity and eventual cell death. The cellular levels of protein, carbohydrate, RNA and DNA were measured in the freshly-prepared suspension and again after six days of starvation at 4°, 20° and 37°C. In addition the extracellular material was analyzed at this time as an indication of potential cellular leakage. It should be noted, however, that the extracellular material is expressed as a function of volume and therefore cannot be quantitatively compared to the decline of cellular constituents which were evaluated on a dry weight basis.

Tables 5 and 7 show the changes in the chemical composition of peptonized milk-grown and synthetic sewage-grown cells that occurred after six days in a non-nutrient environment. Tables 6 and 8 indicate the loss of cellular material to the medium during this period. The response of the organisms to starvation conditions was quite similar as suggested by their patterns of endogenous substrate consumption.

Protein was the major chemical constituent of these cells which is utilized by the organisms whether starved at 4°, 20°, or 37°C. In the peptonized milk-grown cells, the metabolism of 175 µg protein/mg dry wt represented a 24% decline of the protein originally present. At 37°C the cellular protein decreased by 215 µg/mg dry wt and equalled a 33% loss. The synthetic sewage-grown cells also utilized a substantial quantitiy of their cellular protein during starvation. The amount of protein consumed by the cells increased with the temperature of incubation. At the starvation temperatures of 4°, 20° and 37°C the cellular protein

TABLE 5 Change in the cellular composition of Peptonized Milk-grown cells following 1 week starvation in phosphate buffer at 4, 20 and 37°C.

Temp	Time (Days)	Cell component (µg/mg dry wt) Protein	Carbohydrate	RNA	DNA
4°	0	730	100	35	160
	6	555	65	39	100
	% loss	24	35	0	37
20°	0	-	130	40	125
	6	-	70	22	70
	% loss	-	46	45	44
37°	0	660	160	42	120
	6	445	90	9	90
	% loss	33	44	79	25

TABLE 6 Extracellular release of cellular components from Peptonized Milk-grown cells following 1 week starvation in phosphate buffer at 4, 20 and 37°C.

Temp.	Cell component (µg/ml supernatant) Protein	Carbohydrate	RNA	DNA
4°	34	5	0	48
20°	-	31	0	80
37°	29	97	8	240

TABLE 7 Changes in the cellular composition of Synthetic Sewage-grown cells following 1 week starvation in phosphate buffer at 4, 20 and 37°C.

Temp.	Time (days)	Cell component (µg/mg dry wt) Protein	Carbohydrate	RNA	DNA
4°	0	865	97	61	144
	6	761	62	58	138
	% loss	12	36	5	4
20°	0	532	77	60	154
	6	404	39	35	118
	% loss	24	49	42	23
37°	0	742	153	65	234
	6	350	65	5	122
	% loss	53	58	92	48

TABLE 8 Extracellular release of cellular components from Synthetic Sewage-grown cells following 1 week starvation in phosphate buffer at 4, 20 and 37°C.

Temp.	Cell Component (µg/ml supernatant) Protein	Carbohydrate	RNA	DNA
4°	14	0	0	4
20°	12	24	0	29
37°	27	84	10	200

decreased by 12, 24, and 53% respectively. Despite the significant loss of cellular protein, the data in Tables 6 and 8 indicate that only a very small quantity was being lost to the extracellular environment. Therefore protein appeared to be effectively metabolized by the bacteria from both mixed cultures.

Depending upon the nature of the cell suspension the cellular carbohydrate levels varied from 100-160 µg/mg dry wt for the peptonized milk cells and from 80-150 µg/mg dry wt for the synthetic sewage cells. However, the percentage of carbohydrate utilized during starvation was quite constant and relatively independent of temperature. Both resting cell suspensions that were refrigerated at 4°C reduced their carbohydrate by 35% and by 46-49% if stored at room temperature. At 37°C the peptonized milk-grown cells did not exhibit further utilization of their carbohydrate (44% decline) while the synthetic sewage-grown cells lost 58% of their cellular carbohydrate at this temperature. The analyses of anthrone-reactive material in the supernatant liquid revealed an apparent relationship between temperature of starvation and carbohydrate leakage. Negligible amounts of carbohydrate were found at 4°C, however, at 37°C approximately 90 µg/ml supernatant could be detected.

In contrast to both protein and carbohydrate metabolism, RNA was not utilized at 4°C. However, as the storage temperature increased to 20° and 37°C the percentage of the cellular RNA consumed increased markedly to 40-45% and 80-90% respectively. The efficiency of RNA catabolism was indicated by the failure to detect any orcinol-reactive material in the supernatant of either mixed culture at 4° or 20°C. At

37°C the extracellular ribose was not in excess of 10 µg/ml supernatant. Although RNA was found to be readily metabolized, its actual contribution as an endogenous substrate was rather limited since the peptonized milk-grown cells initially contained only 35-40 µg RNA/mg dry wt. Similarly, freshly-harvested cells from synthetic sewage displayed 60-65 µg RNA/mg dry wt.

Cellular DNA also declined during the six days of starvation and as shown in Table 7 the percentage loss increased with temperature. Measurement of the extracellular DNA clearly indicated its accumulation in the medium, especially at 37°C, where 200-240 µg DNA/ml supernatant was detected. This suggested that little if any of the cellular decline of DNA was due to its catabolism.

Tables 9 and 10 record the changes in chemical makeup of medium C-grown cells and in their surrounding buffer after six days starvation. The pattern of utilization of endogenous substrates by the cells grown on medium C was found to be slightly different than what has been described for the peptonized milk-, and synthetic sewage-grown cultures. Protein was again the primary constituent of these cells and comprised the most significant quantity of intracellular carbon assayed. Its catabolism accounted for a loss of 168, 176 and 319 µg of cell material/mg dry wt from cells starved at 4°, 20° and 37°C respectively. Assaying the suspending buffer indicated the decline of cellular protein was due exclusively to its metabolism and not lost through leakage.

Unlike the previous two mixed cultures, the cells generated on the chemically-defined medium C contained only 60-90 µg carbohydrate/mg dry wt. Furthermore, the carbohydrate that was present did not serve

TABLE 9 Change in the cellular composition of Medium C-grown cells following 1 week starvation in phosphate buffer at 4, 20 and 37°C.

Temp.	Time (Days)	Cell component (µg/mg dry wt) Protein	Carbohydrate	RNA	DNA
4°	0	867	60	16	36
	6	699	56	5	76
	% loss	19	7	69	-
20°	0	706	81	25	27
	6	530	76	7	76
	% loss	25	6	72	-
37°	0	638	93	27	16
	6	319	98	3	24
	% loss	50	-	89	-

TABLE 10 Extracellular release of cellular components from Medium C-grown cells following 1 week starvation in phosphate buffer at 4, 20 and 37°C.

Temp.	Cell component (µg/ml supernatant) Protein	Carbohydrate	RNA	DNA
4°	18	17	0	12
20°	0	36	0	3
37°	0	85	0	26

as an endogenous substrate during starvation. Interestingly the supernatant fluid revealed measurable quantities of carbohydrate, especially at 37°C where 8.5 µg/ml was detected.

RNA again proved to be a favoured endogenous substrate in these cells. In contrast to the preceding data for peptonized milk-, and synthetic sewage-grown cultures, however, RNA was almost totally consumed at the 4°C temperature. No extracellular ribose was detected.

Medium C-grown cells did not display cell leakage of DNA during starvation indicating even the non-viable fraction of the population remained intact. Because DNA was measured on a dry wt basis a manifestation of its retention in the cell was seen as an increase in the cellular DNA content after a six day period.

DISCUSSION

DISCUSSION

Ribbons and Dawes (85) have stated that it is meaningless to discuss endogenous metabolism and cell survival without specific reference to the growth environment of the organisms prior to starvation, since these processes are a function of the chemical composition of the cell which is in turn subject to control by the growth medium. It was therefore necessary to evaluate the effect of medium composition on the survival of the mixed cultures. In an attempt to standardize the physical factors (i.e. temperature, aeration, pH etc.) and more importantly the cultural variances (i.e. rate and phase of growth), which are known to affect survival characteristics of bacteria (81), the mixed cultures were grown in the continuous culture system previously described.

A number of methods were used to assess the active biomass during the six day period of starvation. The simplest procedure was to examine the viability of the cell suspension by plate count. The viability retained by medium C-grown cells indicated survival was strongly influenced by the temperature of starvation (Fig. 17a). Reducing the temperature from 37^{o} to $20^{o}C$ increased the survival rate from 7% to 51% which could be further increased to 88% if the cell suspension was refrigerated throughout the six days.

It was also noted that in the resting cells prepared from medium

C and starved at 4° or 37°C, an increased viable count occurred after the third day of incubation. This phenomenon, termed 'regrowth' or 'cryptic growth' is the result of limited cell division occurring among the viable organisms at the expense of nutrient released into the buffer from dead cells. Regrowth has been observed in resting cell suspensions of E. aerogenes (81, 89), Bacterium lactis aerogenes (38) and S. enteritidis (26).

In resting cell suspensions prepared from the synthetic sewage culture the plate count data suggested the survival was very poor even at the lower temperatures (Fig. 17b). Therefore the assumption that storage of resting cells at refrigeration temperatures can significantly prolong their survival may not always be valid. Postgate and Hunter (81) have shown that the optimum survival of E. aerogenes occurred at 20°C and a further lowering of the temperature to 10°C accelerated the death rate of the population. Cook and Wills (20) reported that the survival of suspensions of E. coli was not significantly increased by lowering the temperature from 20° to 4°C.

Although the starvation survival of synthetic sewage-grown cells did not appear to equal the medium C-grown cells, it is believed part of this apparent loss can be explained by the aggregation of cells in the buffer. This was particularly noticeable in the peptonized milk cell suspensions which resulted in erratic data when assessing viability by the spread-plate method. Similar problems of acquiring accurate data by agar plating procedures due to cell aggregation have been mentioned by Strange (92) and by Robertson and Batt (86).

As an alternative approach, the concentration of ATP in the suspension was selected as a parameter of survival since ATP is known to be present only in viable organisms (78) and when an efficient extraction procedure is used the total fraction of viable cells in the suspension, whether present as flocs or as individual cells, will be assayed (62). It was found that the ATP method gave a reasonable impression of the state of the mixed culture during starvation (Fig. 18). Although the relationship between colony formation and cellular ATP as indicators of viability is not clear, the percentage survival in the medium-C suspensions and the peptonized milk suspensions was quite similar at all three starvation temperatures. On this basis it may be proposed that the level of ATP can serve as a useful alternative for assaying the viable biomass of a resting cell suspension.

Reece et al. (84) have provided convincing experimental evidence that correlated the adenylate energy charge in starved suspensions of Peptococcus prevotii to its viability as measured by plate count. On the other hand Strange et al. (91) stated that no direct relationship could be found between ATP levels in E. aerogenes and their survival in resting cell suspension.

The endogenous respiration rate of an organism is indicative of the rate at which its intracellular substrates are being oxidized. The cell suspensions prepared from the peptonized milk and medium C mixed cultures and starved at 20° and 37°C both reached a basal level of endogenous $Q(O_2)$ of 1-2 by the third day (Fig. 19 a, b). However,

the survival of the 20°C-starved cells was substantially better than that found at 37°C (Fig. 17a and 18). These results are in agreement with the observations of Burleigh and Dawes (9) who concluded that the rate at which starved cells respire is not a measure of the ability to grow and divide. The resting cell suspensions starved at 4°C maintained an endogenous $Q(O_2)$ value of at least 5 throughout the six days indicating the low temperature retarded to rate of endogenous metabolism. In the synthetic sewage-grown cells the endogenous $Q(O_2)$ values recorded after the second day of starvation were virtually identical at all three temperatures suggesting an apparent rapid decline in the endogenous reserves (Fig. 19c). The initial rates of endogenous respiration observed in the three mixed cultures are comparable to the values recorded for a number of pure cultures with a wide variation of survival capabilities (9, 21, 27, 86).

While internal substrates can be rather rapidly consumed by the cell, it is apparent that the ability to oxidize exogenous substrates can be retained for a considerable length of time (Fig. 20). The peptonized milk and medium C-grown cells exhibited a linear decline in oxygen uptake at 4° and 20°C but an expoential loss of activity when starved at 37°C. In the resting cell suspensions prepared from both these sources the remaining percentages of the exogenous $Q(O_2)$ after six days starvation were quite similar to the survival measurements based on ATP content or viable plate count. In contrast, the exogenous

$Q(O_2)$ for the synthetic sewage-grown cells after six days at 4^{o} and $20^{o}C$ was considerably higher than would have been expected from the plate count data (Fig. 17b). This conflict reaffirms the danger of underestimating the true viability of a dense cell suspension by the agar plating method which cannot differentiate single cells from cell clumps. In this respect a physiological parameter, like respiratory rate, has a distinct advantage.

Postgate and Hunter (81) noted that the endogenous respiration of E. aerogenes fell much more rapidly than did the cell viability. The respiration rate measured in response to exogenous glycerol, however, paralleled the decline in viability of the cell suspension. Exogenous oxygen uptake has also been correlated to survival in resting cell suspensions of S. lutea (9) and A. crystallopoietes (7, 27). Therefore it can be proposed that exogenous respiration can also be used to estimate the active biomass of a mixed population of resting cells provided a multiple substrate, such as casamino acids, is used.

Chemical analyses were performed on each of the freshly-prepared cell suspensions and again after the six day starvation period in order to determine which cellular components were serving as endogenous substrates and whether the degradation of any one of these components was critical to the survival of the bacteria (Tables 5, 7 and 9). In addition, the analyses of the supernatant buffer indicated whether the loss of a cellular constituent was a result of its metabolism or simply due to cell leakage (Tables 6, 8 and 10).

The data for all three mixed cultures indicated protein was the major cell component accounting for 65-75% of the dry weight in the peptonized milk- and synthetic sewage-grown cells and 80-90% of the dry weight in the cultures generated on medium C. Protein was also quantitatively the most important endogenous substrate at all three starvation temperatures with approximately 15-25% being utilized at 4°C which increased to approximately 35-50% being consumed at 37°C. Analysis of the supernatant liquid found only small quantities of extracellular protein suggesting it had been efficiently metabolized by the cells.

The measurement of the total cellular carbohydrate showed that both the peptonized milk- and synthetic sewage-grown cells possessed a significant reserve of respirable carbohydrate. Approximately 45-50% of the carbohydrate initially present in these cells could be considered respirable since increasing the starvation temperature from 20° to 37°C did not result in an appreciable increase in the percentage of the cellular carbohydrate degraded. The remaining anthrone-reactive material can be accounted for as structural carbohydrate which is known not to serve as an intracellular substrate (81, 85, 98). The medium C-grown cells failed to accumulate any utilizable carbohydrate reserves as indicated by the absence of carbohydrate degradation, even at 37°C. In all three mixed cultures an increasing concentration of extracellular carbohydrate was noted as the starvation temperature rose from 4° to 37°C. It is probably the result of non-respirable

carbohydrate released after cell death and autolysis, knowing that a large percentage of the cells had died by the sixth day of starvation at 37°C.

In the endogenous metabolism of mixed cultures generated on the peptonized milk and synthetic sewage medium, substantial amounts of the protein and carbohydrate reserves were utilized at 4°C. RNA, however, was not consumed at 4°C, yet after starvation at 37°C 80-90% of the cellular RNA had been effectively degraded. Ribose was virtually absent from the buffer at all three temperatures. The results suggest that the lower temperature retarded the consumption of protein and respirable carbohydrate reserves and thereby delayed the initiation of RNA degradation in the cell. The ability of organisms to preferentially degrade carbon-containing polymers in advance of protein or RNA has been reported in E. aerogenes (89), E. coli (24), A. crystallopoietes (7) and Nocardia corallina (86).

In the medium C-grown cells, the failure to accumulate carbohydrate reserves resulted in a more immediate reliance on cellular RNA which was almost totally consumed by the cells at 4°C. The enhanced degradation of protein and RNA in glycogen-poor cells has been noted by Strange et al (89) and by Dawes and Ribbons (24).

Postgate (82) proposed that the degradation of cellular RNA is the critical process in the survival of E. aerogenes. Other investigators, however, have found no correlation between the degradation of any one cellular constituent and the loss of viability in the organism (9, 86, 98). The results for the medium C-grown cells tend to support the latter concept, since the survival of the cells was approximately 90% after six days at 4°C despite a 70% decline in their cellular RNA.

Quantitatively the amount of RNA available as an endogenous substrate was quite limited in all three mixed cultures. Because ribosomes are known to contain the largest reserves of RNA in the cell, it can be assumed that the mixed cultures generated in all three chemostats had relatively little ribosomal RNA (36, 67, 90). Considering the slow growth rate of the cultures ($D = 0.04\ hr^{-1}$), the rate of protein synthesis and hence the requirement for ribosomes would expectedly be minimal. Postgate and Hunter (81) have shown that the survival of resting cell suspensions of E. aerogenes was shortened when the dilution rate of the chemostat used to generate the cells was decreased. They believed the poorer survival of slow-growing cells is related to their low RNA content.

It is generally accepted that while RNA may be effectively degraded during starvation, DNA is not utilized as an endogenous substrate (7, 44, 103). The peptonized milk- and synthetic sewage-grown cells exhibited a substantial loss of cellular DNA. This decline in DNA was accompanied by its accumulation in the suspending buffer, and suggests that as the reserve of protein, carbohydrate and RNA becomes spent, the integrity of the starved cells cannot be maintained resulting in the loss of DNA from the cell. Postgate and Hunter (81) have stated that the structural integrity of E. aerogenes remains intact after cell death with no release of DNA occurring. This also appeared to be true for the cultures generated on medium C as demonstrated by the lack of diphenylamine-reactive material in the buffer, even after six days starvation at $37^{o}C$ when most of the bacteria were no longer viable.

In summary it has been found that the nutrient formulation used to generate the mixed cultures in a chemostat will affect the biochemical composition of the cells and as a consequence may affect the survival capability of the resting cell suspension. The results indicate that protein and carbohydrate are utilized in advance of RNA, although a loss of viability did not parallel its degradation. DNA is not a favoured endogenous substrate and may be lost from the cells through leakage. Endogenous respiration does not relate to cell survival yet exogenous oxygen uptake in response to a substrate is indicative of the active biomass present in the cell suspension. On the basis of this parameter little difference was found between the survival potentials in the three mixed culture peparations. Determination of survival by conventional agar plating may not always be reliable due to cell aggregation and regrowth occurring at high cell densities. The measurement of the ATP level in the suspension appears to be a superior method for the evaluation of viable biomass.

REFERENCES

REFERENCES

1. Barker, S.B. and W.H. Summerson, 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138:535-554.

2. Bashucky, W.S. 1975. Stabilization of selected organic substrates by sewage lagoon bacteria as a function of dissolved oxygen concentration. M.Sc. Thesis, Univ. of Man.

3. Bergter, F. and D. Noak, 1966. Ein mikrobiologisches beispiel fur oszillationen in einer zwei-arten-gesellischaft. Studia Biophys. 1:257-264 (cited from Tempest, 1970).

4. Bissonnette, G.K., Jezeski, J.J., McFeters, G.A. and D.G. Stuart, 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. 29:186-194.

5. Blok, J. 1975. Measurement of the viable biomass concentration in activated sludge by respirometric techniques. Water Res. 10:919-925.

6. Bowen, T.J., Dagley, S. and J. Sykes, 1959. A ribonucleoprotein component of Escherichia coli. Biochem. J. 72:419-425.

7. Boylen, C.W. and J.C. Ensign, 1970. Intracellular substrates for endogenous metabolism during long-term starvation of rod and spherical cells of Arthrobacter crystallopoietes. J. Bacteriol. 103:578-587.

8. Bungay, H.R. and M.L. Bungay, 1968. Microbial interactions in continuous culture. Adv. Appl. Microbiol. 10:269-290.

9. Burleigh, I.G. and E.A. Dawes, 1967. Studies on the endogenous metabolism and senescence of starved Sarcina lutea. Biochem. J. 102:236-250.

10. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-322.

11. Burton, R.M., 1957. Determination of glycerol. In Methods in enzymology. Vol. 1. Edited by S.P. Colowick and N.O. Kaplan. Academic Press Inc., New York. pp. 246-248.

12. Calcott, P.H. and J.R. Postgate, 1972. On substrate-accelerated death in Klebsiella aerogenes. J. Gen. Microbiol. 70:115-122.

13. Calcott, P.H. and J.R. Postgate, 1974. The effects of β-galactosidase activity and cyclic-AMP on lactose-accelerated death. J. Gen. Microbiol. 85:85-90.

14. Campbell, J.J.R., Gronlund, A.F., and M.G. Duncan, 1963. Endogenous metabolism of Pseudomonas. Ann. N.Y. Acad. Sci. 102:669-677.

15. Cappenberg, T.E., 1975. A study of mixed continuous cultures of sulfate-reducing and methane-producing bacteria. Microbial Ecol. 2:60-72.

16. Cassell, E.A., Sulzer, F.T. and J.C. Lamb, 1966. Population dynamics and selection in continuous mixed cultures. J. Water Pollut. Contr. Fed. 38:1398-1409.

17. Cherry, D.S., Guthrie, R.K., and R.S. Harvey, 1974. Temperature influences on bacterial populations in three aquatic systems. Water Res. 8:149-155.

18. Chian, S.K. and R.I. Mateles, 1968. Growth of mixed cultures on mixed substrates. I. Continuous culture. Appl. Microbiol. 16:1337-1342.

19. Chiu, S.Y., Fan, L.T., Kao, I.C. and L.E. Erickson, 1972. Kinetic behavior of mixed populations of activated sludge. Biotechnol. Bioeng. 14:179-199.

20. Cook, A.M. and B.A. Wills, 1958. The use of stored suspensions of Escherichia coli I in the evaluation of bactericidal action. J. Appl. Bacteriol. 21:180-187.

21. Dawes, E.A. and D.W. Ribbons. 1962. Endogenous metabolism of Escherichia coli. Biochem. J. 82:49 P

22. Dawes, E.A. and D.W. Ribbons, 1962. The endogenous metabolism of micro-organisms. Annu. Rev. Microbiol. 16:241-264.

23. Dawes, E.A. and D.W. Ribbons, 1964. Some aspects of the endogenous metabolism of bacteria. Bacteriol. Rev. 28:126-149.

24. Dawes, E.A. and D.W. Ribbons, 1965. Studies on the endogenous metabolism of Escherichia coli. Biochem. J. 95:332-343.

25. Doudoroff, M. and R.Y. Stanier, 1959. Role of poly-β-hydroxybutyric acid in the assimilation of organic carbon by bacteria. Nature, 123:1440-1442.

26. Druilhet, R.E. and J.M. Sobek, 1976. Starvation survival of Salmonella enteritidis. J. Bacteriol. 125:119-124.

27. Ensign, J.C., 1970. Long-term starvation survival of rod and spherical cells of Arthrobacter crystallopoietes. J. Bacteriol. 103:569-577.

28. Fletcher, M. 1977. The effects of culture concentration and age, time and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23:1-6.

29. Gaudy, A.F. and E.T. Gaudy, 1966. Microbiology of waste waters. Annu. Rev. Microbiol. 20:319-336.

30. Gaudy, A.F., Ramanathan, M. and B.S. Rao, 1967. Kinetic behavior of heterogeneous populations in completely mixed reactors. Biotechnol. Bioeng. 9:387-411.

31. George, T.K. and A.F. Gaudy, 1973. Transient response of continuously cultured heterogeneous populations to changes in temperature. Appl. Microbiol. 26:796-803.

32. Ghosh, S. and F.G. Pohland, 1971. Population dynamics in continuous culture of heterogeneous microbial populations. Develop. Ind. Microbiol. 12:295-311.

33. Grady, C.P.L. and A.F. Gaudy, 1969. Control mechanisms operative in a natural microbial population selected for its ability to degrade L-lysine. III. Effects of carbohydrate in continuous-flow systems under shock load conditions. Appl. Microbiol. 18:790-797.

34. Grady, C.P.L., Harlow, L.J. and R.R. Riesing, 1972. Effects of growth rate and influent substrate concentration on effluent quality from chemostats containing bacteria in pure and mixed cultures. Biotechnol. Bioeng. 14:391-410.

35. Grady, C.P.L. and D.R. Williams, 1975. Effects of influent substrate concentration on the kinetics of natural microbial populations in continuous culture. Water Res. 9:171-180.

36. Gronlund, A.F. and J.J.R. Campbell, 1965. Enzymatic degradation of ribosomes during endogenous respiration of Pseudomonas aeruginosa. J. Bacteriol. 90:1-7.

37. Harder, W. and H. Veldkamp. 1971. Competition of marine psychrophilic bacteria at low temperatures. Antonie van Leeuwenhoek. J. Immunol. Serol. 37:51-63.

38. Harrison, A.P., 1960. The response of Bacterium lactis aerogenes when held at growth temperature in the absence of nutrient: an analysis of survival curves. Proc. Roy. Soc. B. 152:418-428.

39. Harte, M.J. and F.C. Webb, 1967. Utilization of mixed sugars in continuous fermentation. II. Biotech. Bioeng. 9:205-221.

40. Hendricks, C.W., 1972. Enteric bacterial growth rates in river water. Appl. Microbiol. 24:168-174.

41. Herbert, D., Elsworth, R. and R.C. Telling, 1956. The continuous culture of bacteria; a theoretical and experimental study. J. Gen. Microbiol. 14:601-622.

42. Herbert, D., Phipps, P.J. and R.E. Strange, 1971. Chemical analysis of microbial cells. In Methods in Microbiology. Vol. 5B. Edited by J.R. Norris and D.W. Ribbons. Academic Press Inc., London. pp. 209-344.

43. Herbert, S.D., Fairbairn, S.R. and M. Davies, 1976. Syntrophic interactions between bacteria isolated from activated sludge. Soc. Gen. Microbiol. Proc. 4:31-32.

44. Holden, J.T., 1958. Degradation of intracellular nucleic acid and leakage of fragments by Lactobacillus arabinosus. Biochim. Biophys. Acta, 29:667-668.

45. Holme, T. and H. Palmstierna, 1956. On glycogen in Escherichia coli B; its synthesis and break-down and its specific labelling with ^{14}C. Acta Chem. Scand. 10:1557-1562.

46. Iannotti, E.L., Kafkewitz, D., Wolin, M.J. and M.P. Bryant, 1973. Glucose fermentation products of Ruminococcus albus grown in continuous culture with Vibrio succinogenes: changes caused by interspecies transfer of H_2. J. Bacteriol. 114:1231-1240.

47. Ingram, I., 1939. The endogenous respiraton of Bacillus cereus. I. Changes in the rate of respiration with the passage of time. J. Bacteriol. 38:599-612.

48. James, A., 1964. The bacteriology of trickling filters. J. Appl. Bacteriol. 27:197-207.

49. Jannasch, H.W., 1967. Enrichments of aquatic bacteria in continuous culture. Archiv. für Microbiol. 59:165-173.

50. Jannasch, H.W., 1968. Competitive elimination of Enterobacteriaceae from seawater. Appl. Microbiol. 16:1616-1618.

51. Jannasch, H.W., 1974. Steady-state and the chemostat in ecology. Limnol. Oceanogr. 19:716-720.

52. Jannasch, H.W. and R.I. Mateles, 1974. Experimental bacterial ecology studied in continuous culture. Adv. Microbial Physiol. 11:165-212.

53. Jost, J.L., Drake, J.F., Fredrickson, A.G. and H.M. Tsuchiya, 1973. Interactions of Tetrahymena pyriformis, Escherichia coli, Azotobacter vinelandii and glucose in a minimal medium. J. Bacteriol. 113:834-840.

54. Kay, C.W., 1977. Kinetics of CO_2 production by intact bacterial resting cell suspensions. Ph.D. Thesis, Univ. of Man.

55. Lamanna, C., 1963. Endogenous metabolism with special reference to bacteria. Ann. N.Y. Acad. Sci. 102:515-793.

56. Lamanna, C. and M.F. Mallette, 1965. Basic bacteriology, 3rd. ed., The Williams and Wilkins Co., Baltimore. p. 691.

57. Larkin, J.M., 1972. Peptonized milk as an enumeration medium for soil bacteria. Appl. Microbiol. 23:1031-1032.

58. Larsen, D.H. and R.L. Dimmick, 1964. Attachment and growth of bacteria on surfaces of continuous culture vessels. J. Bacteriol. 88:1380-1387.

59. Lee, S.S., Jackman, A.P. and E.D. Schroeder, 1975. A two-state microbial growth kinetics model. Water Res. 9:491-498.

60. Lewis, P.M., 1967. A note on the continuous culture of mixed populations of lactobacilli and streptococci. J. Appl. Bacteriol. 30:406-409.

61. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

62. Lundin, A. and A. Thore, 1975. Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. Appl. Microbiol. 30:713-721.

63. Luscombe, B.M. and T.R.G. Gray, 1974. Characteristics of Arthrobacter grown in continuous culture. J. Gen. Microbiol. 82:213-222.

64. Macrae, R.M. and J.F. Wilkinson, 1958. Poly-β-hydroxybutyrate metabolism in washed suspensions of Bacillus cereus and Bacillus megaterium. J. Gen. Microbiol. 19:210-222.

65. Maigetter, R.Z. and R.M. Pfister, 1975. A mixed bacterial population in a continuous culture with and without kaolinite. Can. J. Microbiol. 21:173-180.

66. Mallette, M.F., 1963. Validity of the concept of energy of maintenance. Ann. N.Y. Acad. Sci. 102:521-535.

67. Mandelstam, J. and H. Halvorson, 1960. Turnover of protein and nucleic acids in soluble and ribosomal fractions of non-growing Escherichia coli. Biochim. Biophys. Acta, 40:43-49.

68. Mateles, R.I. and S.K. Chian, 1969. Kinetics of substrate uptake in pure and mixed cultures. Environ. Sci. Technol. 3:569-574.

69. Meadows, P.S., 1971. The attachment of bacteria to solid surfaces. Arch. Mikrobiol. 75:374-381.

70. Meers, J.L. and D.W. Tempest, 1968. The influence of extracellular products on the behaviour of mixed microbial populations in magnesium-limited chemostat cultures. J. Gen. Microbiol. 52:309-317.

71. Meers. J.L., 1971. Effect of dilution rate on the outcome of chemostat mixed culture experiments. J. Gen. Microbiol. 67: 359-361.

72. Meers, J.L., 1973. Growth of bacteria in mixed cultures. CRC Crit. Rev. Microbiol. 2:139-184.

73. Monod, J., 1950. La technique de culture continue; theorie et applications. Ann. Inst. Pasteur. 79:390. (Cited from Tempest, 1970).

74. Munnecke, D.M. and D.P.H. Hsieh, 1974. Microbial decontamination of parathion and p-nitrophenol in aqueous media. Appl. Microbiol. 28:212-217.

75. Novick, A. and L. Szilard, 1950. Description of the chemostat. Science. 112:715-716.

76. Novitsky, J.A. and R.Y. Morita, 1977. Survival of a psychrophilic marine vibrio under long-term nutrient starvation. Appl. Environ. Microbiol. 33:635-641.

77. Parnas, H. and D. Cohen, 1976. The optimal strategy for the metabolism of reserve materials in microorganisms. J. Theor. Biol. 56:19-55.

78. Patterson, J.W., Brezonik, P.L. and H.D. Putnam, 1970. Measurement and significance of adenosine triphosphate in activated sludge. Environ. Sci. Technol. 4:569-575.

79. Pirt, S.J., 1965. The maintenance energy of bacteria in growing cultures. Proc. Roy. Soc. Ser. B. 163:224-231.

80. Pirt, S.J., 1972. Prospects and problems in continuous flow culture of microorganisms. J. Appl. Chem. Biotechnol. 22: 55-64.

81. Postgate, J.R. and J.R. Hunter, 1962. The survival of starved bacteria. J. Gen. Microbiol. 29:233-263.

82. Postgate, J.R., 1967. Viability measurements and the survival of microbes under minimum stress. Adv. Microbial Physiol. 1:1-23.

83. Prakasam, T.B.S. and N.C. Dondero, 1967. Aerobic heterotrophic bacterial populations of sewage and artifical sludge. III. Adaptation in a synthetic waste. Appl. Micrbiol. 15: 1128-1137.

84. Reece, P., Toth, D. and E.A. Dawes, 1976. Fermentation of purines and their effect on the adenylate energy charge and viability of starved Peptococcus prevotii. J. Gen. Microbiol. 97: 63-71.

85. Ribbons, D.W. and E.A. Dawes, 1963. Environmental and growth conditions affecting the endogenous metabolism of bacteria. Ann. N.Y. Acad. Sci. 102:564-586.

86. Robertson, J.G. and R.D. Batt, 1973. Survival of Nocardia corallina and degradation of constituents during starvation. J. Gen. Microbiol. 78:109-117.

87. Shindala, A., Bungay, H.R., Krieg, N.R. and K. Culbert, 1965. Mixed culture interactions. I. Commensalism of Proteus vulgaris with Saccharomyces cerevisiae in continuous culture. J. Bacteriol. 89:693-696.

88. Stanier, R.Y., Doudoroff, M., Kunisawa, R. and R. Contopoulou, 1959. The role of organic substrates in bacterial photosynthesis. Proc. Natl. Acad. Sci. 45:1246-1260.

89. Strange, R.E., Dark, F.A. and A.G. Ness, 1961. The survival of stationary phase Aerobacter aerogenes stored in aqueous suspension. J. Gen. Microbiol: 25:61-76.

90. Strange, R.E., Wade, H.E. and A.G. Ness, 1963. The catabolism of proteins and nucleic acids in starved Aerobacter aerogenes. Biochem. J. 86:197-206.

91. Strange, R.E., Wade, H.E. and F.A. Dark, 1963. Effect of starvation on adenosine triphosphate concentration in Aerobacter aerogenes. Nature, 199:55-57.

92. Strange, R.E., 1967. Metabolism of endogenous constituents and survival in starved bacterial suspensions. Biochem. J. 102:34 P.

93. Sundman, V. and G. Carlberg, 1967. A comment on the nutritional grouping of soil bacteria. Can. J. Microbiol. 13:565-568.

94. Taylor, C.B., 1951. The nutritional requirements of the predominant bacterial flora of the soil. Proc. Soc. Appl. Bacteriol. 14:101-111.

95. Taylor, P.A. and P.J. LeB. Williams, 1975. Theoretical studies on the coexistence of competing species under continuous-flow conditions. Can. J. Microbiol. 21:90-98.

96. Tempest, D.W., 1970. The continuous cultivation of microorganisms. In Methods in Microbiology, Vol. 2. Edited by J.R. Norris and D.W. Ribbons, Academic Press, London. pp. 259-276.

97. Thabaraj, G.J. and A.F. Gaudy, 1969. Effect of dissolved oxygen concentration on the metabolic response of completely mixed activated sludge. J. Water Pollut. Contr. Fed. 41:R322-R335.

98. Thomas, T.D. and R.D. Batt, 1969. Degradation of cell constituents by starved Streptococcus lactis in relation to survival. J. Gen. Microbiol. 58:347-362.

99. Thomas, T.D. and R.D. Batt, 1969. Metabolism of exogenous arginine and glucose by starved Streptococcus lactis in relation to survival. J. Gen. Microbiol. 58:371-380.

100. Titman, D., 1976. Ecological competition between algae: Environmental confirmation of resource-based competition theory. Science. 192:463-465.

101. Umbreit, W.W., Burris, R.H. and J.F. Stauffer, 1957. Manometric techniques. Burgess Publishing Co., Minneapolis.

102. Veldkamp. H. and H.W. Jannasch, 1972. Mixed culture studies with the chemostat. J. Appl. Chem. Biotechnol. 22:105-123.

103. Warren, R.A.J., Ellis, A.F. and J.J.R. Campbell, 1960. Endogenous respiration of Pseudomonas aeruginosa. J. Bacteriol. 79:875-879.

104. Williamson, J.R. and R.E. Corkey, 1957. Citrate determination with citrate lyase and malate dehydrogenase. In Methods in Enzymology, Vol. 3. Edited by S.P. Colowick and N.O. Kaplan. Academic Press Inc., New York. pp. 450-453.

105. Winogradsky, S., 1925. Études sur la microbiologie du sol. I Sur la méthode. Ann. Inst. Pasteur. 39:299-254. (cited from Ensign, 1970).

106. Yeoh, H.T., Bungay, H.R. and N.R. Krieg, 1968. A microbial interaction involving combined mutualism and inhibition. Can. J. Microbiol. 14:491-492.

107. Zevenhuizen, L.P.T.M., 1966. Formation and function of the glycogen-like polysaccharide of Arthrobacter. Antonie van Leeuwenhoek J. Microbiol. Serol. 32:356-372.