Growth of Streptococcus bovis and a Butyrivibrio in batch and continuous culture and the relationship of molar growth yield to intermicrobial competition

A thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Microbiology at Massey University

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1974

ABSTRACT

Cell growth yield of Streptococcus bovis and Butyrivibrio were determined in batch cultures where growth was separately limited by glucose, CO_2 and trypticase. With S. bovis, glucose limited growth and a Yg of 39.6 g / M in the presence of excess CO_2 was determined. S. bovis grew in the absence of CO_2, but the Yg was reduced to 16.5 g / M. In the presence of excess CO_2, the Yg determined for Butyrivibrio was 55 g / M. Butyrivibrio was strictly limited by CO_2 and the Y_{CO_2} was equal to Yg. This led to the suggestion that CO_2metabolism allows the generation of at least two additional ATP when combined with glucose metabolism for both organisms.

Monod growth constants were determined for both organisms in continuous culture under glucose limitation. Ks and μ_{max} for S. bovis were 0.429 mM / 1 and 2.47 hr^{-1}, respectively. For Butyrivibrio, Ks and μ_{max} were 0.332 mM / 1 and 0.704 hr^{-1}, respectively. The cell growth yields for S. bovis and Butyrivibrio were determined to be 39.6 g / M and 69.1 g / M, respectively. At growth rates less than 0.2 hr^{-1} colony forming units and total cell counts of S. bovis decreased, but cell yield did not. Colony forming units, total counts and cell growth yield of Butyrivibrio did not decrease at low growth rates.

When S. bovis and Butyrivibrio were grown in continuous mixed culture, Butyrivibrio dominated at growth rates below 0.5 hr^{-1} and growth of S. bovis was strongly depressed. That Butyrivibrio dominated mixed cultures supports the proposition that an organism deriving more ATP per mole of substrate that another will dominate in environments comparable with continuous culture. The roles of maintenance energy, Ks and μ_{max} and cell yield in competition are considered.

ACKNOWLEDGEMENTS

Dr. B. D. W. Jarvis, for much useful advice

Mr. A. G. McCulloch for all methods of scintillation counting and autoradiography

Dr. R. T. Clarke, for providing the bacterial cultures

Mr. P. P. Roche, for assistance with the glassblowing and entertainment

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Inroduction

The bovine rumen is a semi-continuously fed culture which contains a wide variety of protozoa and bacteria. These microorganisms obtain their energy and nutrients by fermenting the food eaten by the animal and the animal in turn obtains energy by absorbing the end products of the microbial fermentation and digests the microorganisms themselves. Volatile fatty acids which cannot be metabolized anaerobically in the rumen are absorbed by the ruminant and metabolized aerobically to provide energy for the synthesis of glucose (Hungate, 1966). The microorganisms are digested in the abomasum and duodenum and constitute a major source of protein for the animal.

The stoichiometry of the rumen fermentation has been a topic of interest because of its direct implications in the bovine nutrition. Approximately 75 % of the available carbohydrate is converted to fatty acids, which are utilized by the animal (Barcroft, et al, 1944). That microbial protein synthesis is capable of supporting ruminant growth has been shown (Loosli, et al, 1949; Virtanen, 1966). The actual amount of protein available to the cow in the form of microbial cells has been estimated by a number of means but the precise amount remains indefinite, chiefly due to the difficulty of separating the microbes from the other rumen contents (Walker & Nader, 1968). The 10 g of microbial protein synthesized per 100 g of carbohydrate estimated as maximum by Hungate (1966) has been increased by later authors using label incorporation (Walker and Nader, 1968; Al-Rabbat et al, 1971; Pilgrim, et al, 1970) and phospholipid synthesis (Bucholtz & Bergen, 1973). From these increases has come the suggestion that the average yield of cell material per hexose, and hence the number of ATP derived from each hexose, in the rumen should be increased.

Butyrivibrio fibrisolvens is a rumen organism that ferments carbohydrates to

CO_2, H_2, ethanol, and acetic, butyric, formic and lactic acids (Hungate, 1966). It is a common rumen organism which usually occurs in the rumen at a concentration of at least $10^8 ml^{-1}$ (Bryant & Burkey, 1953). The production of significant quantities of butyric acid and the numbers present in the rumen indicate that Buyrivibrio contributes significantly to metabolism in the rumen (Bryant & Small, 1956).

Streptococcus bovis is also a rumen organism, which is not normally found in the ruminant diet. Although S. bovis can always be isolated from rumen contents, its numbers seldom exceed $10^7 ml^{-1}$, and its main fermentation product is lactate (Hungate, 1966). It has been shown that the lactate pool in rumen contents is normally small and turns over slowly (Jayasuriya & Hungate, 1959). Consequently, S.bovis has been considered an organism not contributing greatly to ruminant metabolism (Hungate, 1966). However, conversion of lactate to volatile fatty acids in whole rumen contents has been demonstrated (Nakamura & Takahashi, 1971) and lactate may be considered a normal intermediate in the rumen fermentation.

Under certain conditions, when the ruminant diet is shifted from low to high carbohydrate, the production of lactic acid can increase to such an extent that acid indigestion results due to the inability of the digesta to metabolize lactic acid as rapidly as it is produced. When this occurs, the concentration of S.bovis is found to have increased to the vicinity of $5 \times 10^9 ml^{-1}$ and is considered one of the most significant contributors to acid indigestion. The ability of S.bovis to generate such high numbers appears to be due to its high maximum specific growth rate (a doubling time of 20 minutes).

The fact that S.bovis has a high maximum rate of growth and yet normally exists in low numbers has been explained in two ways. In animals shifted to high grain diets, high concentrations of S.bovis occur for a period of time, but in well adapted animals, the numbers are similar to those found in animals receiving a low grain diet. This has been

attributed to the establishment of a new equilibrium population in which it is possible that the S. bovis serve as food for an enlarged protozoan population. An alternative suggestion for the normally low concentration of S. bovis has been its poor ability to compete due to its relatively inefficient energy yielding metabolism in contrast to other rumen bacteria.

It has been generally accepted that established pathways of energy metabolism generate predictable yields of high energy intermediates, such as ATP, and that these are used to synthesize new cell material with constant efficiency. This constant, as determined by Bauchop & Elsden (1960) is 10.5 g per mole of ATP. This constant has been verified for a variety of microorganisms (Forrest & Walker, 1971) and used widely for its predictive value. In 1966, Hungate suggested that "..... in the competition to achieve maximum growth, selection is against lactic acid and ethanol production. Formation of each of these products entails loss of available ATP. Conversion of pyruvate to acetyl CoA and the reduction of acetyl CoA to ethanol similarly entails loss of a potential ATP. The acetyl-CoA can yield an ATP unless it must be used for hydrogen disposal... according to this view, the propinionate formed in the rumen represents additional synthesis of ATP. This is also true for acetate on the hypothesis that pyruvate is split to acetyl CoA. A production of ATP in butyrate formation has been surmised, but efforts to demonstrate it have been unsuccessful. If there is a selection for maximum biochemical work, butyrate should also represent an end product accompanying additional energy conservation by the cell.

According to these views, the carbon dioxide, methane, acetate, propionate and butyrate, final products in the rumen fermentation, are formed because pathways leading to them provide the most efficient conversion of fermentable substrate into microbial cells."

The purpose of the research reported in this thesis was to test the proposition that an organism which derives a relatively low yield of two ATP per mole of glucose fermented will not be able to compete with an organism which can derive additional ATP by metabolism of pyruvate to acetate, propionate or butyrate. Two organisms were chosen for the experiments, S. bovis, which is a homofermenter and was expected to derive only two ATP per mole of glucose (Hungate, 1966) and Butyrivibrio fibrisolvens, which produces primarily formic and butyric acid and was expected to derive a larger number of ATP per mole of glucose fermented.

Continuous culture must be used to study competition based on substrate utilization because the outcome in batch culture will depend simply on the maximum specific growth rate and the duration of the lag phase for each organism. The organism with the larger maximum specific growth rate and shorter lag phase will always dominate if growth of both organisms is limited by the same substrate. Continuous culture in a chemostat allows this type of competition to be studied by limitation of the culture to a specific growth rate. Both organisms will have the same specific growth rate, and assuming equal affinity for substrate, dominance will depend on the efficiency with which the organism utilizes the substrate to synthesise new cell material.

The equations of continuous culture were originally derived by Monod (1950) and subsequently by Herbert et al (1956). Equations of significance to the work in this thesis are the following:

A. Dependance of specific growth rate on substrate concentration

$$\mu = \mu_{max} S / (K_s + S) \qquad \text{equation (1)}$$

μ = specific growth rate

μ_{max} = maximum specific growth rate

K_s = substrate concentration at which $= \frac{1}{2} \mu_{max}$

S = substrate concentration

B. The inverse of equation (1) provides a graphical means of determining the constants μ_{max} and K_s. A plot of equation (2) is called a Lineweaver-Burke plot.

$$1 / \mu = 1 / \mu_{max} + (K_s / \mu_{max})\ 1 / S \qquad \text{equation (2)}$$

C. Molar growth yield

$$Yg = x / (Sr - S) \qquad \text{equation (3)}$$

Yg = grams cells produced per mole of substrate consumed

x = dry weight of cells in grams per liter

Sr = original or reservoir concentration of substrate

S = final or growth vessel concentration of substrate

Variations on these equations (Van Uden, 1969; Powell, 1967) due to the inconstancy of Yg and the occurrence of maintenance metabolism will be considered in the discussion in regard to the results shown in Section III of this thesis. Awareness that these variations can occur led me to measure parameters necessary for the determination of viability (total and colony forming units (CFU) counts) and yield (dry weight) in continuous culture experiments.

II.1. Medium: Constituents and Preparation

II.1.1. Standard medium used in all experiments was derived from Medium 10 of Caldwell and Bryant (1966). The constituents per 100 ml of the medium were as follows: mineral solutions (A & B), 5.0 ml each; $Na_2CO_3.H_2O$, 0.4 g; Trypticase (BBL), 0.2 g; yeast extract (Difco), 0.05 g; cysteine hydrochloride, 0.025 g; indigo carmine, 0.001 g; agar (Davis), 2.0 g. Two mineral solutions were prepared in order to prevent precipitation of phosphate salts. These solutions had the following composition (g/l):

Solution A: K_2HPO_4, 5.92; NaCl, 0.888; $(NH_4)SO_4$, 9.0; $CaCl_2.6H_2O$, 1.794; $MgSO_4.7H_2O$, 1.872

Solution B: KH_2PO_4, 3.54

To obtain differential counts and to study the substrate specificity of the two strains, other carbohydrate substrates were substituted for glucose in the standard medium.

When tubed medium was being prepared, the methods described by Hungate (1969) were followed with the exception that solid cysteine.HCl was added to the bulk medium immediately prior to tubing instead of adding a solution of cysteine.HCl to each tube just before inoculation.

Liquid medium was tubed in 10 ml quantities after it had been equilibrated to 39 C. to avoid problems of low or high internal pressure when making transfers by syringe.

Solid medium was prepared by adding agar to the standard liquid medium, bringing the mixture to the boil, adding cysteine.HCl and dispensing in 5 ml quantities into standard roll tubes.

All tubed media were autoclaved at 121 C., 72 kilo-Pascals, for 15 minutes.

Cultures of Streptococcus bovis (hereafter referred to as Sb1) and a strain tentatively identified as Butyrivibrio (hereafter referred to as WV1) were provided by Dr. R. Clarke (Applied Biochemistry Division, DSIR, Palmerston North, New Zealand). Stock cultures of these bacteria were

maintained on standard liquid medium with at least a weekly subculture. Upon original receipt of the cultures, several subcultures of each bacterium were made, incubated overnight, quick-frozen in liquid air and stored at -70 C. Every two or three months, stock liquid cultures were replaced by deep frozen cultures.

II.1.2. Preparation of Medium for Continuous Culture

Medium for Continuous culture was prepared in a 10 litre boiling flask. All constituents but glucose and cysteine.HCl were mixed and bubbled with O_2-free CO_2 for 10 to 20 minutes. Immediately prior to autoclaving, solid cysteine.HCl was added. A loose cover (cheesecloth) was placed over the top and the medium was autoclaved for 30 minutes at 121 C. and 72 kilo-Pascals. Less than 30 minutes autoclaving gave incomplete reduction of the medium. Immediately after autoclaving, the medium was transferred to a water bath at 39 C. and equilibrated under a sterile stream of O_2-free CO_2. Due to loss of medium volume in the autoclave, it was necessary to prepare an additional 1.5 litres of medium which was used to re-adjust the volume to 10 litres after equilibration of both lots to 39 C. Just before adjusting the volume of the medium, a sterile O_2-free aqueous glucose solution (10 g / 10 ml) was added to the boiling flask from a roll tube. This tube was rinsed by syringe with two 10 ml volumes of medium (containing no glucose). As the glucose was added through the same port as the medium used to adjust the volume, quantitative transfer of glucose into the 10 litre reservoir was assured. The contents of the flask were mixed and equilibrated with O_2-free CO_2 for six hours before use.

II.2. Batch Culture Apparatus

II.2.1. Standard Roll Tubes

Standard 16 x 150 mm roll tubes (Bellco Glass Inc., Vinland, New Jersey, USA) closed with size '00' recessed butyl rubber stoppers (Hungate, 1969) were used for routine purposes, including maintenance of stock cultures and colony count determinations.

II.2.2. Nephelometer Vessels

When it was necessary to follow growth densitometrically, it was necessary to use specially made tubes to satisfy conditions of anaerobic growth and fit into the Klett-Somerson densitometer. Lengths of pyrex glass tubing of 15 mm o.d. were made into 11 cm tubes, closed at one end and constricted at the other to fit a 00 butyl rubber stopper. For following the growth of larger volumes of culture, 140 ml vessels were made by connecting an 11 cm length of 15 mm o.d. pyrex tubing to one end of a 12 cm length of 38 mm o.d. thick walled (1.5 mm) pyrex tubing and closing the other end with a short tube into which a '00' butyl rubber stopper could be fitted. The smaller tubes were used in ^{14}C labelling experiments in which it was necessary to collect samples just as the stationary phase of the growth cycle was reached. The larger flasks were used when cell dry weights were to be determined. Both vessels could be autoclaved while sealed without fracturing.

II.2.3. Large Batch Cultures

Batch cultures with volumes greater than 50 ml were grown in Erlenmeyer flasks. The flasks were sealed with rubber stoppers pierced by four glass tubes. Two tubes just penetrated the stopper, one being used for inoculation and the other for a CO_2 exhaust. The other two extended to the bottom of the flask as shown in figure 1A. This arrangement allowed collection or transfer of anaerobic samples from continuously bubbled cultures.

Figure 1-A: Anaerobic head for Erlenmeyer Flask

a.) Butyl rubber tubing

b.) O_2-free CO_2input

Figure 1-B: Water Trap

Figure 1-C: Head for 10 Litre Medium Reservoir used for Continuous Culture

a.) CO_2 exhaust

b.) Port for Addition of Glucose

c.) O_2-free CO_2 input

d.) Butyl Rubber Tubing

e.) Medium Outflow

f.) Quickfit 34/35 Male Joint

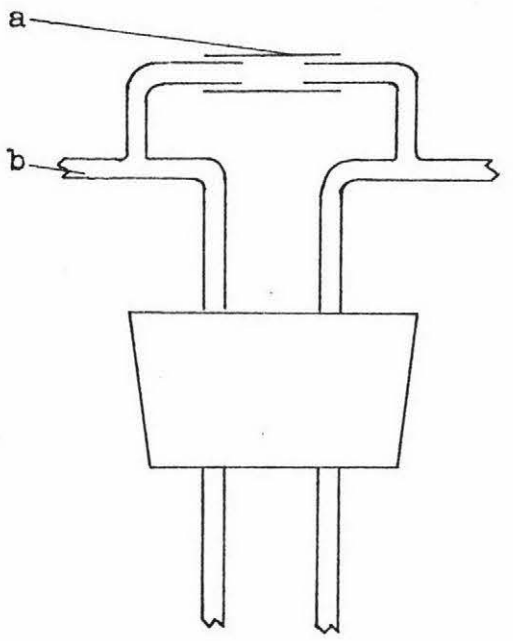
Figure 1-D: Medium and CO_2 input to growth vessel of Continuous Culture

a.) Medium Input

b.) O_2-free CO_2 input

c.) Quickfit 19/26 Male Joint

A



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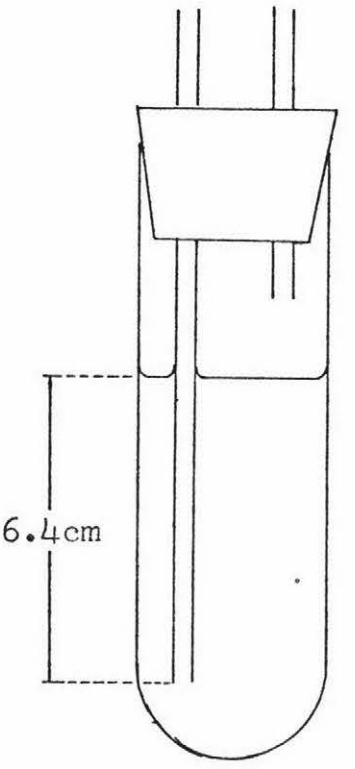

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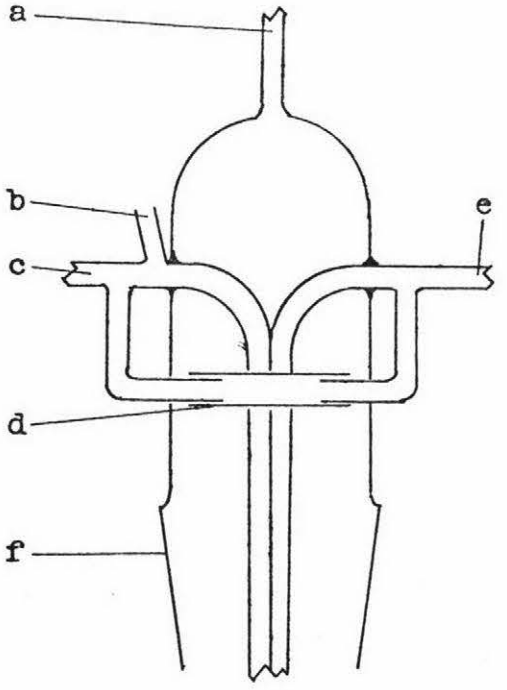

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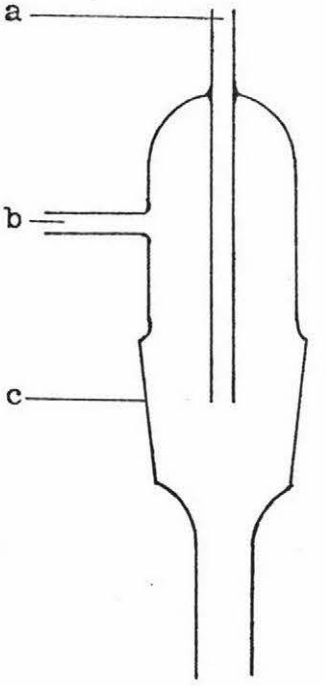


II.3. Continuous Culture Apparatus

A diagram of the assembled continuous culture apparatus is shown in Figure 2. Elements of the system are described below.

II.3.1. Carbon Dioxide System

Commercially obtained carbon dioxide was passed through a hot copper column at 380 C to remove trace amounts of oxygen. Two columns were arranged in parallel to allow continual flow of CO_2 to the growth vessel in the event that one column became oxidised during an experiment.

The copper columns were made of 1 inch i.d. pyrex glass tubing. The tube was wrapped in asbestos tape and then wound with a 6.5 ft. of 4.1 ohm per foot nichrome wire. The wire was covered with a fine layer of asbestos tape and then two layers of coarse asbestos tape. A glass post made of 5.0 mm thick glass rod was fused to the glass tubing of the column near the exit end. This rod penetrated the asbestos tape to allow observation of the copper packing. When the copper turned dark in one column, the gas flow was diverted through the alternate column, and the first column was reduced with H_2gas. The heating coils of the two columns were connected in series and power was supplied by a 5 amp variable transformer (A. Gallenkamp & Co., Ltd., London, EC 2.). The temperature of the copper in each column was measured with a pyrometer (Gallenkamp, PX040) connected to a thermocouple (Gallenkamp, PX052) placed inside the column. A temperature of 320 to 420 deg. C. was obtained when the transformer was set to deliver a potential of 170 V. The variation in temperature was due to fluctuation of the line voltage between 210 and 250 V.

The rate of flow of CO_2 to the growth vessel was measured with a gas flow meter and maintained at 50 ml / min. This gives a gas volume dilution rate of approximately 3.75 turnovers per hour.

Figure 2: Diagram of Continuous Culture Apparatus

 , Screw Clamp; —>— , Direction of Flow; —— CO_2 Lines;

——, Liquid Lines; ═══ , Electric Lines.

A.) Reduced Copper Columns; B.) Water Bath; C.) Medium Reservoir;

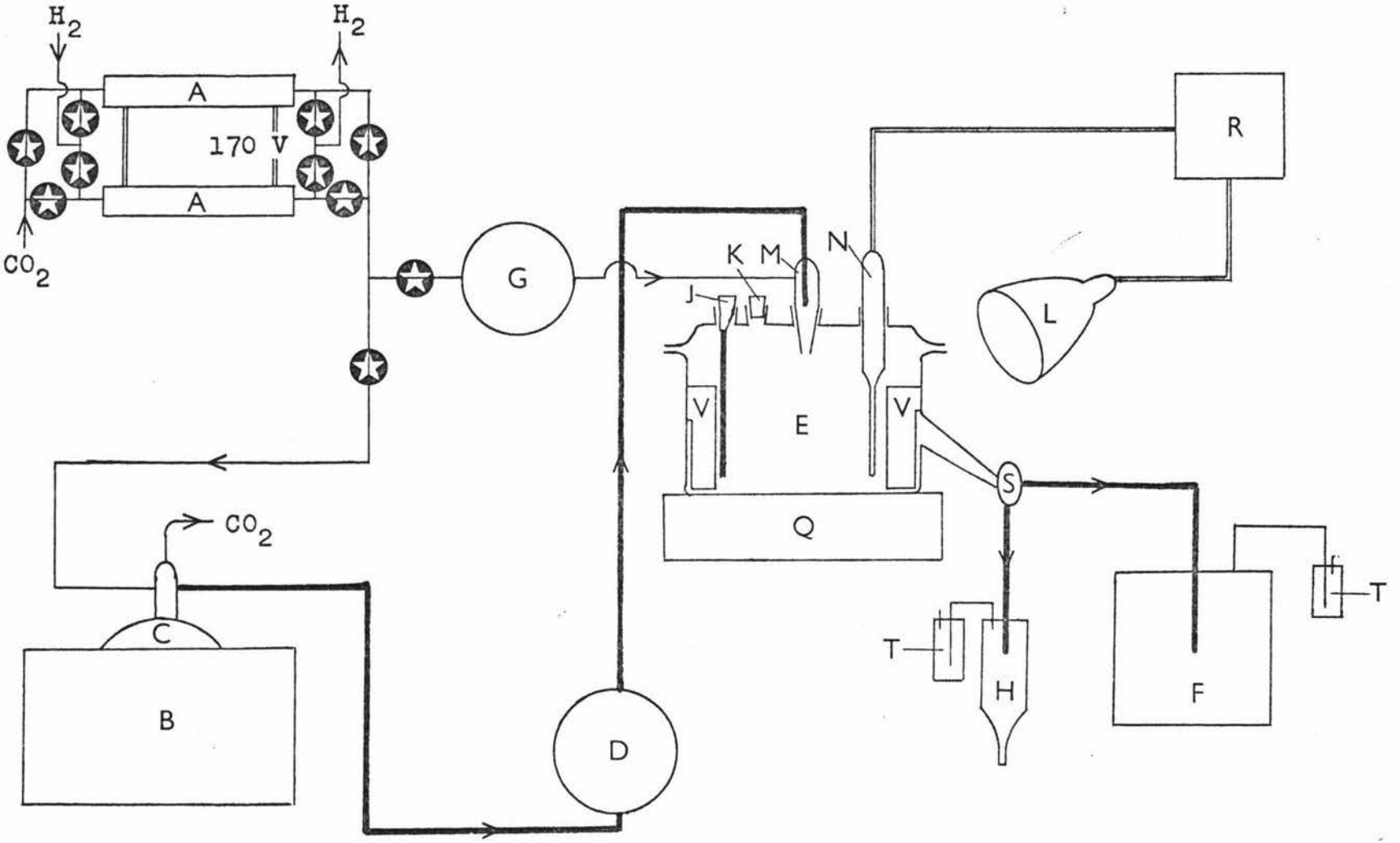
D.) Pump; E.) Growth Vessel; F.) Effluent Reservoir; G.) Gas Flow Meter;

H.) Sample Collection Reservoir; J.) Direct Sampling Port;

K.) Inoculation Port; L.) Infra-red Lamp; M.) Medium and CO_2 input (Fig. 1-D);

N.) Contact Thermometer; Q.) Magnetic Stirrer; R.) Electronic Relay;

S.) Three Way Stop-cock; T.) Water Trap (Fig. 1-B); V.) Vane of Baffle


H2
H2
A
170 V
A
CO2
G
K
M
N
J
R
L
V
E
V
S
Q
CO2
C
B
D
T
H
F
T

An internal pressure of 5 mm Hg was maintained by having a water trap 6.4 cm in depth at the CO_2 exhaust (Fig. 1-B).

II.3.2. Medium Reservoir

The medium reservoir was made from a 10 litre boiling flask with a female 34 / 35 quickfit joint fixed to the top. The head fitting into this was made from a 34 / 35 male quickfit joint which was constricted to an exhaust at the top and contained three tubes which extended to the bottom of the boiling flask. One of these tubes was stoppered where it penetrated the glass joint and served for the removal of old or unwanted medium from the reservoir without interrupting an experiment. The other two tubes were arranged as shown in fig. 1-C. These tubes allowed CO_2 rinsing of medium delivery lines prior to passage of medium to the growth vessel. During continuous culture, the butyl rubber hose connecting these tubes was clamped off, so that CO_2 entered the flask by the left hand tube and medium passed to the growth vessel by the right hand tube (fig. 1-C).

II.3.3. Pump

The pump used was a variable stroke positive displacement pump("Micro Metering Pump, Series II", F.A. Hughes & Company, Ltd., Blenheim Road, Longmead, Epsom, Surrey, England, UK). This consists of a 1/15 horsepower fan-cooled induction motor and a reduction gearbox. A series of different capacity ranges can be obtained by attaching interchangeable pump heads with different piston diameters to this pump unit. These pump heads may have a short or long stroke mechanism unit. The volume delivered by each pump head is adjusted by varying the piston stroke with a micrometer adjustment. In addition, a capsule reduction unit may be interposed between the drive unit and any pump head to give a 5:1 reduction in the rate of piston movement.

Initially a size 2 pump head with a long stroke mechanism (capacity 750 ml/hr)

was used with a porcelain piston. A stainless steel piston (size 2) was used subsequently due to repeated breakage of porcelain pistons during fitting of the sterilized pump head to the long stroke mechanism. This stainless steel piston suffered wear to the extent that leakage was continuous and this allowed the entrance of contaminants. Eventually, the size 2 pump head and long stroke mechanism were replaced with a size 4 pump head and a short stroke mechanism (capacity 925 ml/hr) fitted with a porcelain piston. This arrangement provided breakage free assembly and the piston showed no signs of wear after several months of use.

In conjunction with the reducing gear, the larger head provided a range of pumping rates of up to 0.7 hr^{-1}, which was suitable for the slower growing bacterium. Without the reduction gear, dilution rates of up to 3.0 hr^{-1} were available and these were appropriate for the faster growing organism. Numerous rate measurements gave constant values once the rate was set.

In order to comply with the requirement stated in the pump manual for a 3 ft. delivery and a flooded suction head, the medium reservoir was placed on the floor next to the pump, and the tubing subsequent to the pump head extended to a height of five feet above the pump head before emptying into the growth vessel. The medium reservoir was maintained at 39°C. to avoid the formation of CO_2 bubbles in the pumping head.

II.3.4. Temperature Control

Temperature was controlled by use of a contact thermometer (Gallenkamp, TM-480, 0-111 deg. C.). This thermometer provided the triggering switch for an electronic relay (Gallenkamp, EC-780) which powered a 250 Watt infra-red lamp (Phillips IR 133-2B/479*) directed onto the growth vessel.

No difficulties were experienced with the system in two years of use.

II.3.5. Growth Vessel

The growth vessel was made from a "Quickfit" culture vessel (Quickfit, FV1L) of one litre capacity. It was 10 cm i.d. and 16 cm in depth. The top of the growth vessel was a Quickfit multi-socket/flat flange lid (Quickfit, MAf/41). The joint between lid and flask was smeared with silicone grease and clamped with a wire clip (Quickfit, JC100F).

The lid had five female Quickfit joints attached to it. The central 19/26 joint was used for medium and CO_2 input (fig. 1-D). The 29/32 Quickfit on the lid was straightened to allow the contact thermometer to reach into the liquid without interfering with the stirring bar. Thermometer passed through a red rubber stopper which was pressed firmly in place during use. An inoculation port composed of a '00' butyl rubber stopper fitted inside a male 14/23 Quickfit joint was inserted in one of the female 14/23 Quickfit joints on the lid.

A sampling tube was made of a glass capillary which extended to the bottom of the flask. The upper end of the capillary passed through a small red rubber stopper which fitted in the lower end of a 14/23 male Quickfit joint. The upper end of the 14/23 male Quickfit joint was constricted to hold a '00' half-bored butyl rubber stopper. The upper end of the capillary was flared and pressed firmly against the butyl rubber stopper. This allowed a syringe needle to pass through the butyl rubber stopper and, guided by the flare into the centre of the capillary, to withdraw samples. The first 2-3 ml of culture withdrawn was always discarded to allow for the unmixed space in the capillary.

The growth vessel was stirred by a 2.0 cm teflon coated stirring bar driven by a magnetic stirrer (CENCO) beneath the growth vessel. Formation of a stable vortex was prevented by use of stainless steel baffles. The baffles were formed from a single sheet of 1/32 inch stainless steel. Four vanes were made to extend in from the sides of the chamber at equal intervals

around the circumference at a distance of 2 mm from the walls. The vanes were 10 mm wide and extended vertically from the bottom of the vessel to well above the surface of the liquid.

The volume of culture in the growth vessel was controlled by a fixed outlet in the side of the growth vessel at a height of 3.8 cm from the bottom of the growth vessel. The total volume of liquid maintained by this means was 270 ml.

Sample collection for colony forming units, Thoma cell counts and glucose were made via the sampling assembly. Larger volumes of culture, used for the estimation of dilution rates and for the collection of dry weight samples, were collected over known times from the growth vessel outflow. The vessel used to collect these samples was made of a 250 ml dropping funnel (Quickfit, QD 12/250). Growth in the collection vessel was inhibited by the addition of 20 ml of 40% formalin per 180 ml of culture collected.

II.4. Analytical Methods

II.4.1. Glucose

Three assays were evaluated for the measurement of glucose in the standard growth medium used in these studies. Two of these, Dreywood's anthrone method (Morris, 1948) and the phenol-sulfuric acid method (Launer, 1963) were abandoned because of the high blank values and wide variations between duplicate samples which were obtained when they were used.

The Somogyi-Nelson reagent (Nelson, 1944) gives an absorption with standard medium of 0.130 at 500 nm when read against a water plus reagent blank. This absorption is reduced to about 0.025 if the medium is first treated with a barium sulfate protein precipitation (5 ml medium, 2 ml $BaCl_2$ (0.405M), 2 ml NaOH (0.66M), 1 ml $ZnSO_4$ (0.878M), added and mixed in that order, centrifuged at 10,000 g for 10 min, and the supernatant stored at -20 C. for analysis). It is necessary to run a standard curve with each set of analyses, due to slight variations in the background colour of different batches of medium. A typical standard curve is shown in figure 3.).

All optical densities were measured against a water plus reagent blank in a Beckman DB spectrophotometer using cells with a 1 cm light path. All the results reported are the average of determinations on duplicate samples.

II.4.2. Lactic Acid

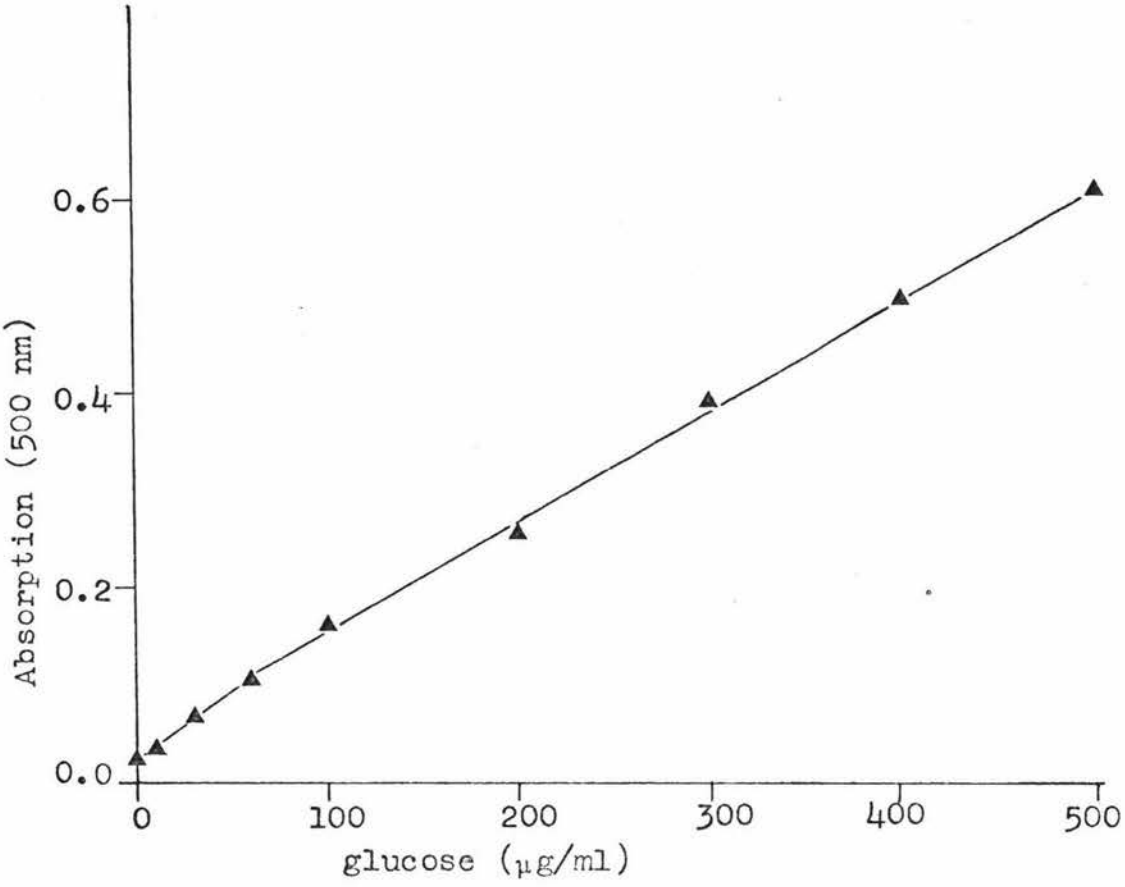
Lactic acid was estimated by the method of Steinsholt and Calvert (1960). The applicability of this method to the measurement of lactate in the standard medium was tested by addition of standard lactic acid (Analar grade, British Drug Houses, Ltd.) to the standard medium to give concentrations ranging from zero to 15 mM/1. Aqueous solutions were prepared in the same concentrations, and both sets of samples were assayed. Plots of optical

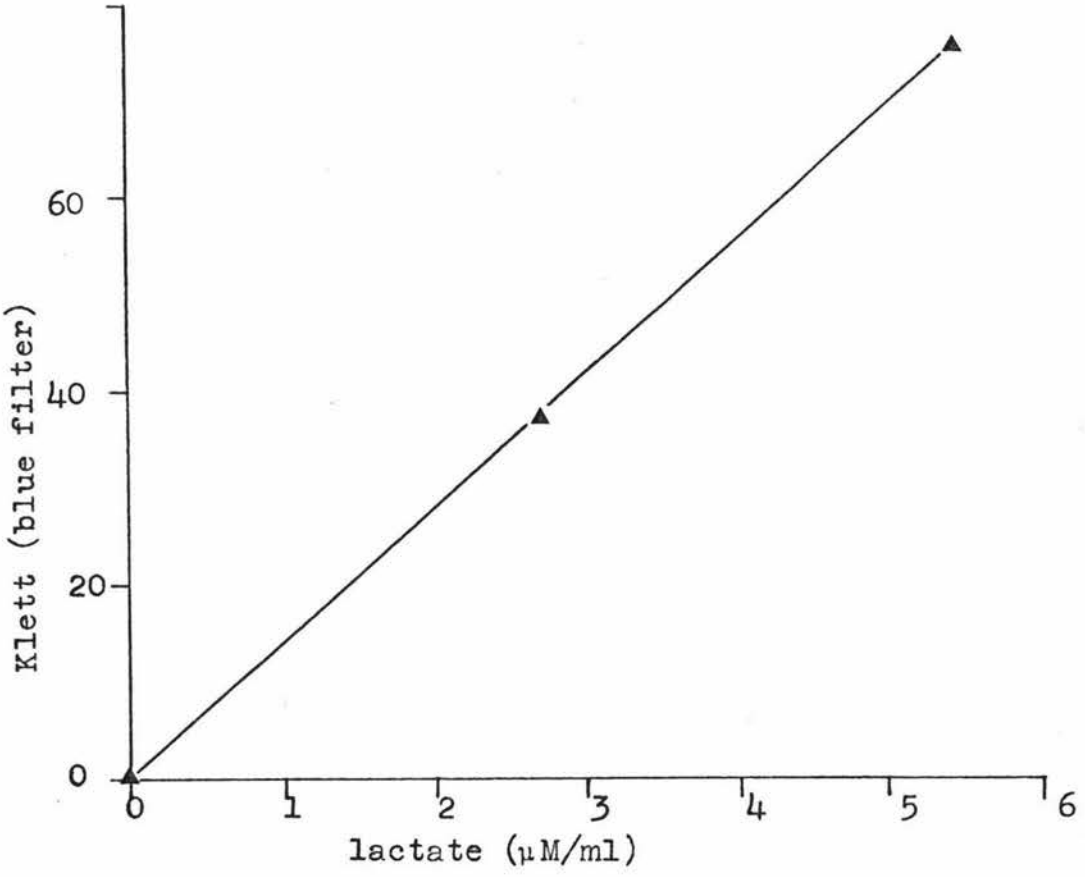
Figure 3: Standard Curve for Glucose Determination

Glucose was determined by the method of Somogyi-Nelson in supernatants of protein precipitates of medium containing known amounts of glucose and read against a water plus reagent blank

Figure 4: Standard Curve for Lactate

Fig.4 shows a standard curve for known lactate concentrations in water as determined by the method of Steinshold and Calbert. Standard medium containing no added lactate usually gave a reading of 10 Klett.


Absorption (500 nm)
0.0
0.2
0.4
0.6
0
100
200
300
400
500
glucose (µg/ml)


Klett (blue filter)
0
20
40
60
0
1
2
3
4
5
6
lactate (µM/ml)

density against concentration for both sets of samples show two parallel lines, with the aqueous sample line passing through the origin and the medium sample line having a positive intercept. The assay of lactic acid was corrected for this by use of a medium blank in all lactic acid assays. A typical standard curve is shown in figure 4.

The optical densities were measured in a Klett-Summerson densitometer (Model 800-3) with a blue filter. Reported values are the average of duplicate determinations.

II.4.3. Volatile Fatty Acids

II.4.3.1. Thin layer chromatography of fermentation products

Volatile fatty acids were qualitatively determined by thin layer chromatography on silica gel (Drucker and Melville, 1966). Drucker and Melville used layers of silica gel 0.25 mm thick, but it was found that under these conditions, the distance travelled with time varied and there was a tendency for samples near the edges to trail those in the center. The running time had to be increased from the recommended 16 hr to 20 hr to obtain adequate separation of formate and acetate.

When the thickness of the gel was increased to 1 mm, separation was improved, the distance travelled with time was more consistent, and no trailing at the edges occurred.

II.4.3.2. Determination of Steam Volatile Fatty Acids

Volatile fatty acids (VFA) were steam distilled in a Markham still (Markham, 1943) and estimated by titration (Neish, A. C., 1952). It was necessary to boil the distilled water used in the steam generator for at least a half an hour before beginning to distill samples containing VFA. High titrations were obtained even when no sample had been added to the still if the water in the steam generator had been recently refreshed. In one instance, the titration decreased to a constant background level only after collection of about 500 ml of steam from a volume of 3.0 l of distilled water in the steam generator.

The samples were added to the still in the following order: 3.0 ml of the culture supernatant, 1.0 ml of 10 $\underline{N}$ H_2SO_4 saturated with $MgSO_4$, and rinsed in with 1.0 ml distilled water. 150 ml of distillate was collected and titrated with 0.01 $\underline{N}$ NaOH (calibrated with weighed standard 0.01 $\underline{N}$ oxalic acid in CO_2-free water). Further 50 ml volumes of distillate collected from the same sample showed no further titrable material. Reported values are the average of duplicate determinations.

II.4.4. Determination of Cell Dry Weight

For batch cultures of 50 ml, dry weights were estimated by centrifuging a 35 ml aliquot of culture at 10,000 g for 10 minutes, washing once and quantitatively transferring the pellet to a pre-weighed milk bottle top. The milk bottle top containing sample was dried to constant weight in a vacuum desiccator under an infra-red lamp and reweighed. The dry weight of residue in mg was multiplied by 28.6 to give the dry weight of cells in mg/l.

Cell dry weights from continuous culture were determined by collecting 180 ml of culture outflow in a graduated dropping funnel containing 20 ml of 40% formalin. It was necessary to shake the dropping funnel at intervals during collection to ensure mixture of the culture with the formalin. The formalised sample was centrifuged in 250 ml plastic bottles, washed once, and weighed as above. The dry weight of residue in mg was multiplied by 5.55 to give the dry weight of cells in mg/l.

II.4.5. Determination of Colony Forming Units

Colony forming units were determined in roll tubes containing 5.0 ml of solid medium. Standard dilutions were carried out with plastic disposable tuberculin syringes which had been rinsed with O_2-free CO_2. The dilution medium was the same as standard liquid medium except for the omission of glucose, and it was equilibrated to 39°C. before use. It was necessary to equilibrate diluent to 39°C. before tubing in order to avoid difficulties in transfer due to pressure differentials. The agar roll tubes were inoculated with 0.5 ml of a known dilution, gently inverted ten times to assure complete mixing and rolled horizontally under cold water to give an even spread of agar over the walls of the tube. Tubes containing Sbl were counted after 24 hours of incubation. Tubes containing WV1 could be counted after 48 hours of incubation, but were normally counted after 72 hours of incubation.

Reported results are normally the mean of three to six replicates from the same dilution. Variation in counts of Sb1 rarely exceeded 10% of the mean, but that of WV1 frequently exceeded 20%. The high variability of WV1 counts may have been due to its sensitivity to oxygen. Frequently, as a result of oxidation of the roll tube or the admission of oxygen during transfer, no growth of WV1 would occur, or growth would occur, after extended incubation, only in the thicker layers of agar near the rubber stopper. Tubes which showed no growth, or growth only near the stopper, were excluded from the average.

A selective medium was used to determine colony forming units of WV1 when Sb1 and WV1 were grown in mixed culture. Growth of Sb1 was excluded by use of xylose instead of glucose as energy source in standard medium. In a test case, WV1 produced as many colonies on xylose as on glucose.

The number of colony forming units of Sb1 in mixed cultures was determined after 24 hours incubation on standard glucose agar, where colonies of WV1 were excluded by their very small size in comparison with Sb1.

II.4.6. Direct Microscopic Count of Cells

Originally a Petroff-Hausser bacterial counting chamber was used to make direct microscopic counts. Counting of bacteria in this cell was very difficult due to evaporation of the suspending medium at the edges of the cover slip with consequent drift of the bacteria across the field of view. Efforts to reduce field drift by increasing the viscosity of the suspending medium by adding various percentages of glycerol or methylcellulose gave no improvement.

The use of a Thoma cell successfully avoided the problem because the sample surface is not in contact with the general atmosphere. The majority of counts reported were made with the Thoma cell. All samples consisted of 3 ml of culture plus 2 ml of 40% formalin. Duplicate samples were collected and

counted as soon as possible. Twenty squares of the Thoma grid were counted for each sample and the mean of both samples was multiplied by 0.333×10^8 to give numbers of cells per ml. The 95% confidence limits were estimated from the standard deviation of all the squares counted according to the equation, $ts/n^{\frac{1}{2}}$, where n=40 and t=2.02 and s is the standard deviation. This figure is multiplied by the same factor as the mean to give the reported 95% confidence limits. Individual cells in chains of cells were not counted, hence the reported values are minimum total estimations.

II.4.7. Radiometric Methods

II.4.7.1. Counting of Samples and Method of Converting Counts per Minute (cpm) to Disintegrations per Minute (dpm)

All radioactive samples were counted in glass vials in a Packard Tricarb (Model 3375) scintillation counter on standard 14-C settings. Each vial was counted twice for ten minute intervals. Counts were corrected for quenching by the channels ratio method (R. J. Herberg, 1965; C. T. Peng, 1965). A conversion curve relating % efficiency of counting to channels ratio was prepared by counting samples of known specific activity with different channels ratios (i.e., differing amounts of quenching). These samples were prepared by adding ^{14}C-hexadecanone of known specific activity to 9 ml of scintillation fluid (see below) and adding quenching compounds to cover as large a span of channels ratios as possible. Quenching compounds used were water, ethanol, chloroform and methyl ethyl ketone. The accuracy of the resultant conversion curve was assumed to be 1.0%, although, at low channels ratios (0.1 or less), where background becomes significant, the error would be greater. The actual error at a channels ratio of 0.75 was determined as $\pm$1.3%. Although the channels ratio and 14-C-channel counts for duplicate counts of the same vial may vary considerably, corrections made from the curve to absolute activity (dpm, including the correction for background dpm)

fell within the accepted criterion of variation (summed standard deviations of channels counts from the data printout of the scintillation counter plus a standard 1.0% conversion accuracy) in 90 to 95% of the instances, which is an acceptable confidence limit.

II.4.7.2. Methods of Sample Collection for Scintillation Counting.

II.4.7.2.1. Collection of Cells

Cells were collected for scintillation counting by membrane filtration. Aliquots (1.0 ml) of 14-C labelled culture were added to a 2 cm diameter, 0.22 micrometer pore size membrane filter (Millipore Corporation, Bedford, Mass. 01730, USA) under suction, and washed twice with 1.0 ml portions of cold medium (not containing glucose). The filter was then removed from the filter holder and dried flat under an infra-red lamp. After five to ten minutes, it was dry and was placed directly into a vial containing 9.0 ml scintillation fluid. Each determination of radioactivity associated with cells is based on the average dpm observed on duplicate samples prepared in this way.

II.4.7.2.2. Collection of Gasses

Gaseous fractions from ^{14}C-labelled cultures were collected by rinsing the entire atmosphere from above a culture with a slow stream of O_2-free N_2 gas through a train of two tubes containing 10 ml of 18% aqueous ethanolamine. In all cases, the second tube contained only background activity.

II.4.7.2.3. Collection of TLC Spots

Spots from thin layer chromatograms were developed in the normal manner and then scraped off the plate with a sharpened spatula. The silica gel thus removed was suspended in water (1.0 ml if from a 0.25 mm thick gel and 2.0 ml if from a 1.0 mm thick gel) and centrifuged at 10,000 g for 5 minutes. Aliquots of 0.5 ml of the supernatant were mixed with the scintillation fluid and stored for counting.

II.4.7.3. Scintillation Fluid and Sample Addition to Scintillation Vials

The scintillation fluid was of the following composition: redistilled sulfur-free toluene (May & Baker), 1 litre; 2,2-p-phenelenebis (5-phenyloxazole) (POPOP) (Sigma Chemical Corporation), 0.300 g; 2,5-diphenyloxazole (PPO) (Sigma Chemical Corporation), 3.00 g; Triton X-100 (BBH), 500 ml.

In order to obtain clear solutions with channels ratios in the range of the conversion curve, samples and scintillation fluid had to be added to the vials in the orders shown below.

a.) alkaline samples
   1. 9.0 ml scintillation fluid
   2. 0.5 ml water
   3. 0.5 ml 18% aqueous ethanolamine sample

b.) aqueous samples
   1. 1.0 ml aqueous sample
   2. 9.0 ml scintillation fluid

c.) membrane filter samples
   1. 9.0 ml scintillation fluid
   2. freshly dried membrane filter

II.4.8. Autoradiography

II.4.8.1. Hydrolysis of Cells

Cells were submitted to an acid hydrolysis by the following procedure (Smith, London & Stanier, 1967). Five ml aliquots of ^{14}C-labelled cells were centrifuged at 4000 g for ten minutes. The supernatant was discarded and the pellet was resuspended in chilled medium and recentrifuged. The supernatant was discarded and the pellet was suspended in 5 ml of boiling 70% ethanol. The alcoholic suspension was centrifuged at 200 g for ten minutes and the supernatant was discarded. The pellet was suspended in 1.0 ml of H_2O.

This was then transferred to a vial containing 1.0 ml of concentrated HCl (Pronalys, BDH) to give a final concentration of 6 N HCl. The vial was sealed under suction and autoclaved for at least twelve hours (121 deg.C, 72 kilo Pascals).

II.4.8.2. Thin Layer Chromatography of Acid Hydrolysates

Thirty micro-litres of acid hydrolysate were spotted onto 0.1 mm thick layers of cellulose powder (MN300, Macherey & Nagel) on 20 x 20 cm square glass plates. These were then chromatogrammed first into 100 parts phenol: 40 parts water (w/v) (Basham & Calvin, 1957). The phenol was redistilled according to Draper & Polard (1949). After drying, the plate was run at right angles to the phenol direction in a solvent composed of 47 parts n-butanol: 23 parts n-propanol: 30 parts water (v/v/v).

Two spots of differing size of radioactive blue ink were applied to the plates above the solvent fronts to coordinate the plates and the developed film. These plates were then applied face down to the film surface of 8 x 10 inches Osray M (Agfa-gevaert) X-ray film, and stored in the original film packet for nine weeks. Amino acids were qualitatively identified by ninhydrin spray (Data for Biochemical Research, 1969).

III. Results

III.1. Batch Culture Results

III.1.1. Batch Culture of Sb1

III.1.1.1. General Cultural Characteristics

Sb1 is a gram positive coccus. It usually appears in pairs in liquid batch culture, but may form chains up to six cells in length. Cellular size is usually consistent at about 1 micro-meter diameter. The cell size gradually reduced as the culture aged. With a fresh inoculum, growth is usually visible in liquid culture within four hours. A red pigment is produced, but its intensity varies. Occasionally, when Sb1 was frequently subcultured, pigment intensity would increase dramatically. As a pigmented culture aged, the pigment settled to the bottom of a tube and, if shaken, eventually disappeared.

Colonial morphology in glucose agar roll tubes is shown in plate 1. Subsurface colonies are disc shaped. Surface colonies are circular with a convex surface and an entire edge. Surface colonies tended to run down the inner face of the agar with extended incubation. The colour of the colonies may vary from cream white to deep rusty red, but the colour is usually a moderate rusty red. The frequency with which deeply coloured colonies appeared increased at higher growth rates, as observed in colony counts from continuous culture.

Growth with a number of substrates is shown in table 1. All substrates were at 0.1 % (w/v). Where growth appeared, it was approximately to the same optical density.

Several efforts were made to demonstrate the presence of capsular or reserve material with Sb1. Reserve material stains attempted were Albert's stain for volutin, Sudan Black B stain for lipid, and alcian blue stain for polysaccharide (Norris, J.R. & Swain, H., 1971). None of these stains were positive for Sb1. Negative staining for capsular material was attempted with nigrosin with rose bengal, and nigrosin with carbol fuchsin.

Plate 1: Surface and subsurface colony of Sb1

Plate shows a small subsurface colony (lenticular shaped) connected to a large surface colony. Magnification ca x 13.

Plate 2: Capsule of Sb1

Plate shows a phase contrast photomicrograph of a wet mount of Sb1 suspended in sonicated india ink. Magnification ca x 1000.

Plate 5: Sub-surface multi-lobed colony of WV1

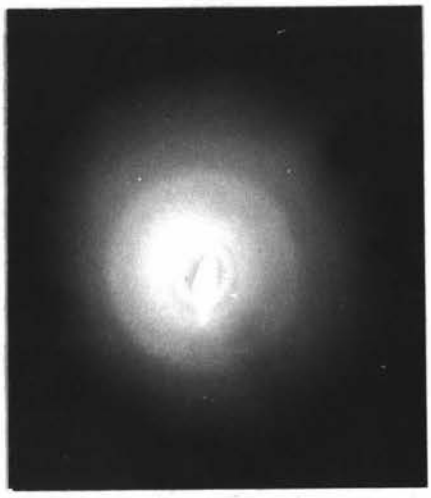
Magnification ca 25 x.

Plate 6: Complete surface colony of WV1

Magnification ca x 25.

Plate 7: Rhizoid surface colony of WV1

Surface colony of WV1 shows the rhizoidal characteristic. Subsurface portion is almost visible as a disperse ball.
Magnification ca x 20.



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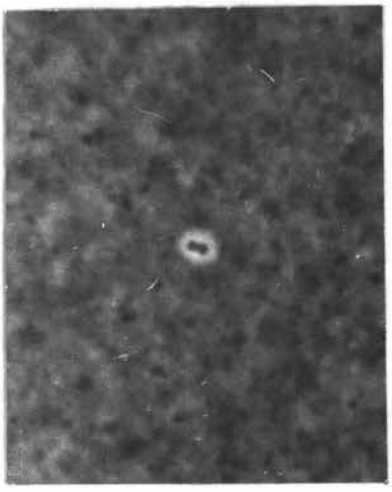

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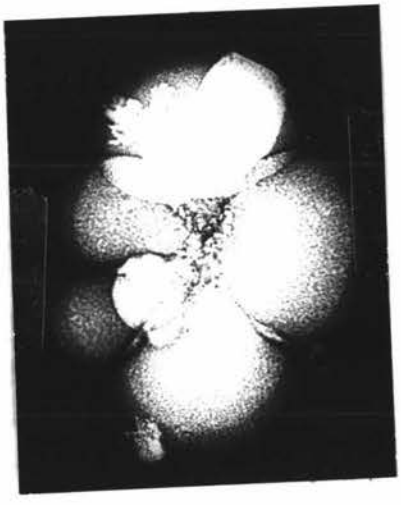

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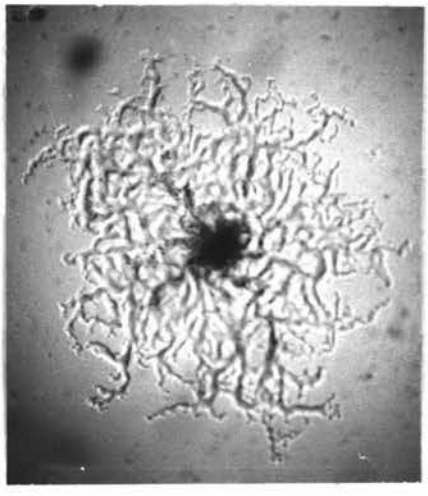

7

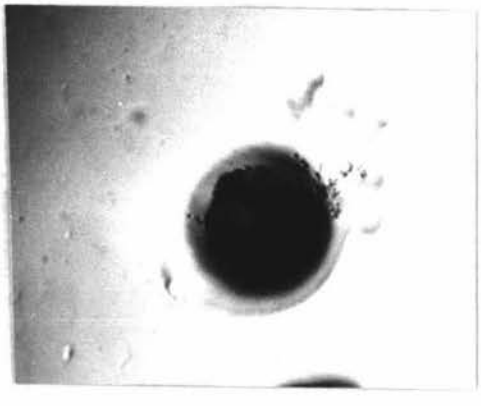

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Table 1: Growth of Sb1 and WV1 with a variety of substrates

Substrate	Sb1	WV1
Blank	-	-
D(+)fucose	-	-
L(+)arabinose	-	+
D(-)fructose	+	+
D(+)galactose	+	+
lactose	+	+
D(+)mannose	+	+
D(+)melibiose	+	+
rhamnose	-	-
soluble starch	+	+
D(+)trehalose	+	-
xylose	-	+
glucose	+	+
cellobiose	+	+
lactate	-	-

All cultures were grown in standard liquid medium with glucose replaced by the respective compounds shown. Each culture was inoculated with 0.1 ml of an overnight culture of the respective organism and incubated for five days before discarding.

An indefinite positive result was obtained as the capsular material was not extensive, and the cell did not stain strongly. The best demonstration of the presence of a capsule was by phase contrast examination of a wet mount in sonicated india ink (Pelikan) (plate 2). With this preparation, Sb1 appears as a dark oval surrounded by a phase bright ring equal in thickness to the cell diameter.

The classification of Sb1 as Streptococcus bovis was partially confirmed by a number of tests designed to distinguish between streptococci of Lancefields group D (Fackham, R., 1972). The distinguishing results were the following: 1.) acid production in litmus milk 2.) absence of growth in 6.5 % NaCl 3.) clot formation in litmus milk 4.) no hydrolysis of gelatin 5.) hydrolysis of starch 6.) production of extra-cellular polysaccharide on 5 % sucrose broth.

III.1.1.2. Growth Yield of Sb1 with Glucose

A large number of experiments were performed where growth yield of Sb1 with varying levels of glucose was measured. A summary of all these experiments, where dry weights for each level of glucose are an average, is shown in figure 5. From this curve, the range of glucose concentrations that are limiting growth can be seen to extend from zero to about thirty milli-molar glucose. The slope of the line over this region (Yg) is 40.2. At no level of glucose where growth is measurable as rate of change in optical density is there a reduced rate of growth. Levels of glucose above forty milli-molar were inhibitory to growth.

A single experiment demonstrated many points of growth with glucose for Sb1 (figure 6). In that experiment, the range of glucose concentration was zero to eighty milli-molar. The cultures were inoculated with Sb1 and the change in optical density was followed in the Klett. The doubling time was 0.32 hour (19.2 minutes, a specific growth rate of 2.16 hr^{-1}) for all

Figure 5: Batch Growth of Sb1: Cell Yield with Glucose

Dry weights are shown as mg/L. Each point is the average of at least two experiments. The line is an unweighted regression of the points plotted for Glucose up to 40 mM/L. The slope of this line (Yg) is 40.2.

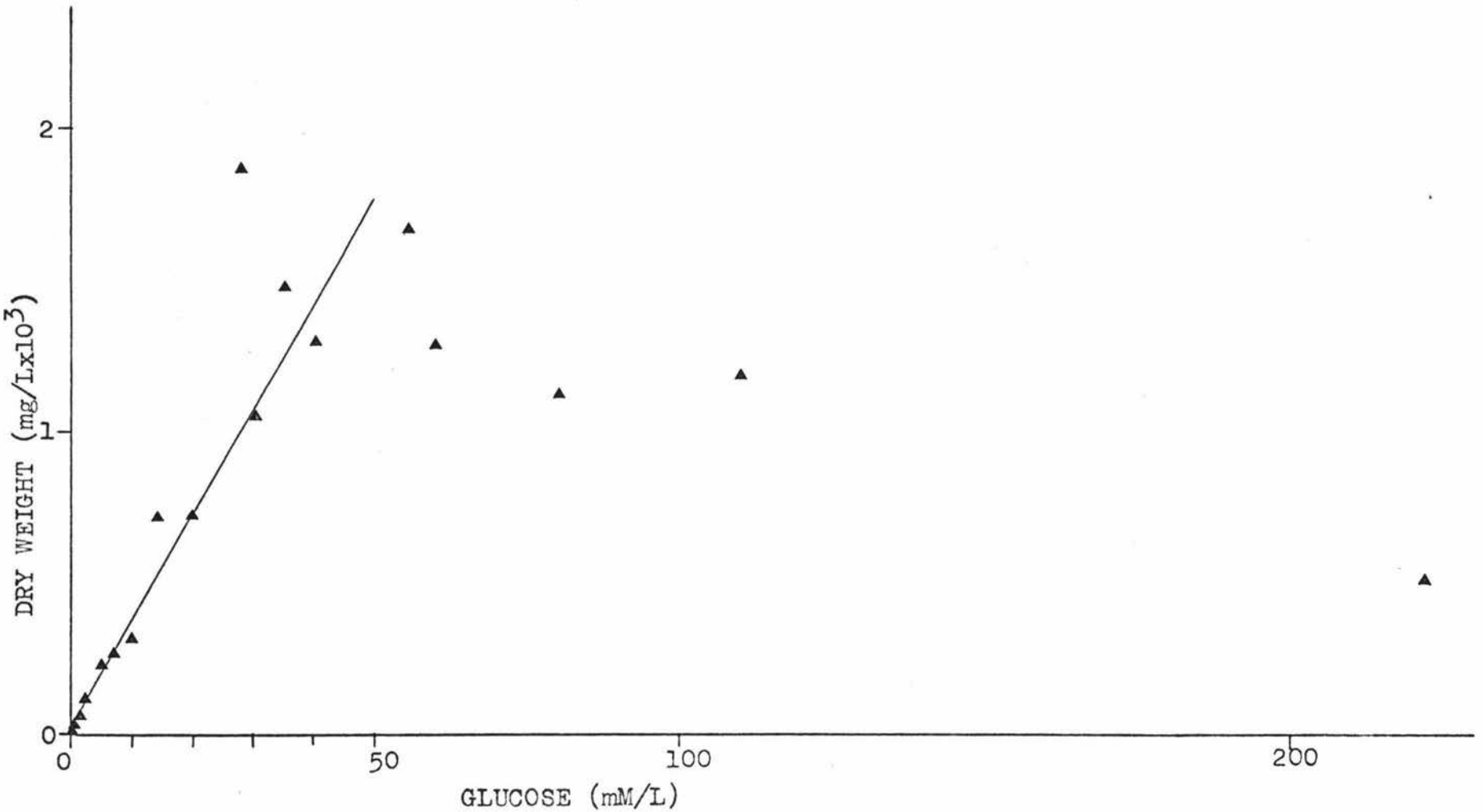
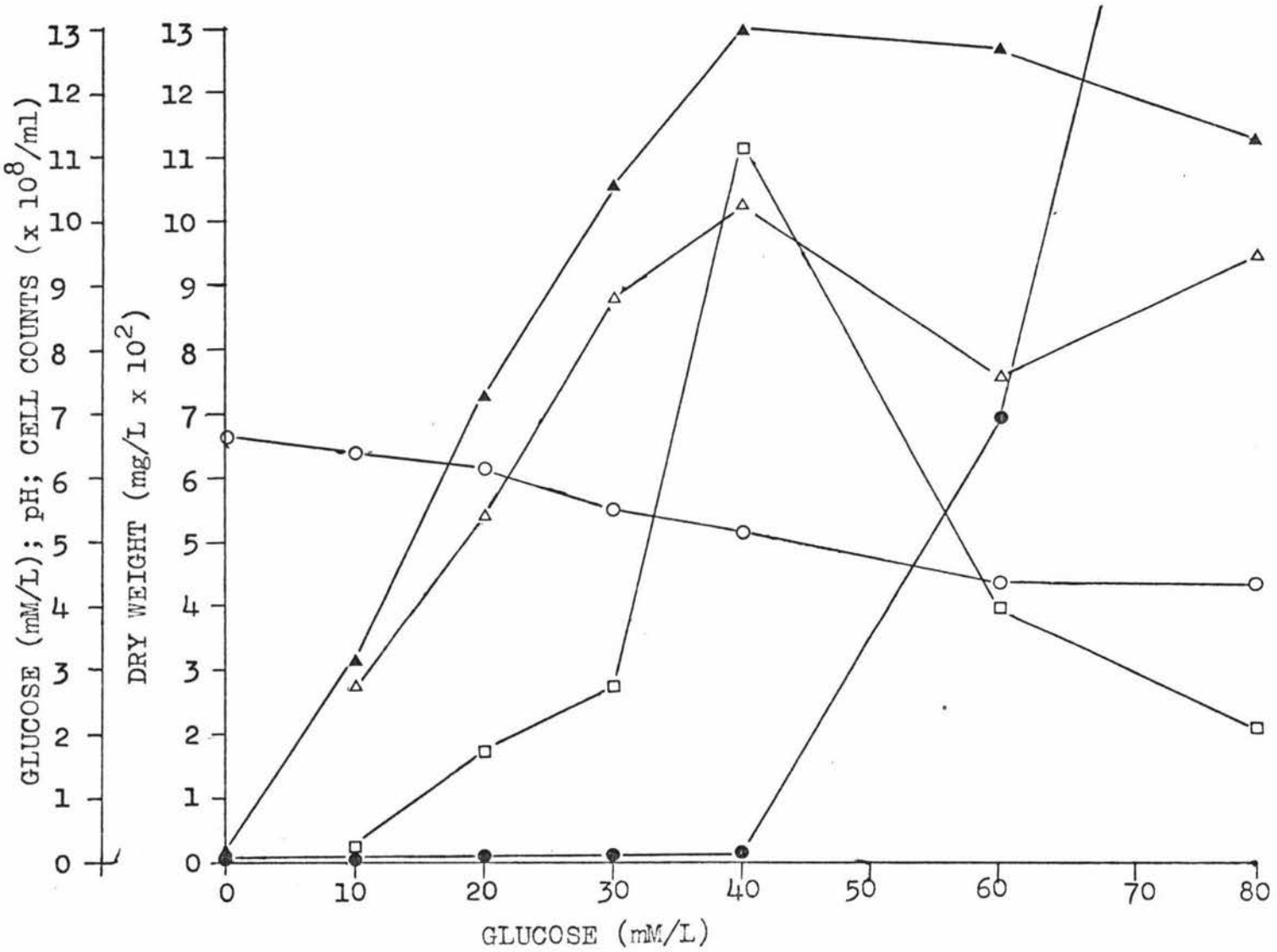

DRY WEIGHT (mg/Lx10^3)
2
1
0
0
50
100
200
GLUCOSE (mM/L)

Figure 6: Batch Growth of Sb1: Cell Yield and Other Parameters of Growth with Glucose

Figure 6 shows several growth measures of Sb1. All factors were measured at the beginning of stationary phase of batch cultures of Sb1 in nephelometer flasks. ▲ , dry weight; △ , cell numbers; □ , pigment production; ● , glucose remaining at the beginning of stationary phase; ○ , pH at stationary phase.


GLUCOSE (mM/L); pH; CELL COUNTS (x 10^8/ml)
DRY WEIGHT (mg/L x 10^2)
GLUCOSE (mM/L)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0
10
20
30
40
50
60
70
80

cultures. Dry weight and direct count of cells increased linearly up to forty milli-molar glucose and then began to decrease at higher glucose concentrations. The final pH dropped linearly over the range zero to sixty milli-molar glucose from pH 6.7 to pH 4.3. At higher levels of glucose, the pH went no lower than 4.3. Almost all glucose was consumed up to forty mM/L, above which the amount of glucose remaining at the end of logarithmic growth rose rapidly. The red pigment was measured by measuring the optical density of the aqueous wash of the dry weight samples in the Klett with a blue filter. By this measure, the amount of pigment roughly paralleled the dry weight of cells until levels of glucose above forty milli-molar were reached, when it decreases more rapidly than the dry weight. A difference spectrum using the forty milli-molar supernatant with the thirty millimolar supernatant as blank showed a single broad absorption peak at 400 to 410 nano-meters (figure 7).

III.1.1.3. Growth Yield of Sb1 with Tryticase

In order to observe the effect of trypticase and yeast extract concentration on growth yield of Sb1, batch cultures were grown in standard medium at 5.55 mM glucose with varying levels of trypticase and yeast extract. Trypticase and yeast extract were maintained in a constant ratio of 4 : 1, respectively, as in the standard medium. The medium was prepared by addition of trypticase, yeast extract and 0.0125 gram cysteine hydrochloride dry to 140 ml nephelometer flasks, gassing with carbon dioxide for 15 minutes, and adding 50 ml equilibrated medium less trypticase and yeast extract. Immediately after addition of medium, the flasks were sealed and autoclaved (15 minutes, 72 kN/m^2). The results of this experiment are shown in figure 8.

The growth rate is almost maximal at 2.0 grams trypticase per liter (the level of trypticase in standard medium). A Lineweaver-Burke type plot of equation 2 where trypticase is S, gives a Ks of 0.0465 gram trypticase

Figure 7: Visible Absorption Spectrum of Pigment Produced by Sb1.

The spectrum was measured using supernatant from culture grown at 40 mM/L glucose as sample and culture supernatant grown at 20 mM/L glucose as reference.

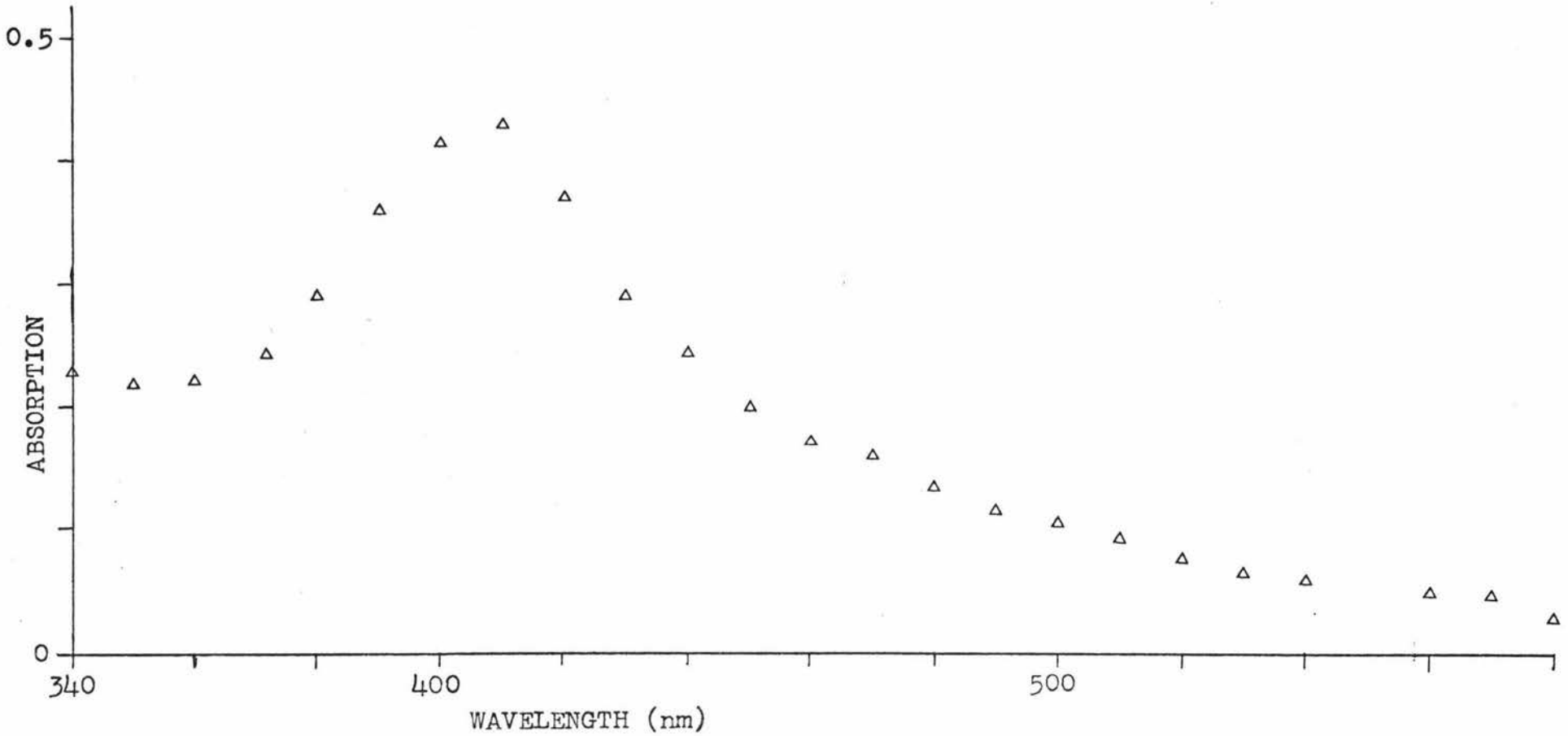

0.5
0
ABSORPTION
340
400
500
WAVELENGTH (nm)

Figure 8: Growth of Sb1 with Tryticase Culture of Sb1 were grown on standard medium at 5.55 mM/L glucose with specific additions of trypticase as shown. o , Specific growth rate; Δ , dry weight.

SPECIFIC GROWTH RATE (hr^{-1})
0
1
2
DRY WEIGHT (mg/L x 10^2)
0
1
2
3
4
5
6
TRYPTICASE (g/L)
0
2
10
20

per liter.

Above 2 grams trypticase per liter, the increase in dry weight is approximately linear with an extrapolated intercept of 250 milli-grams per liter at zero tryticase concentration. This gives a Yg estimation of approximately 45 grams per mole at zero trypticase concentration.

III.1.1.4. Growth Yield of Sb1 with Carbon Dioxide

Carbon dioxide is known to affect the growth of many rumen micro-organisms (Dehority, 1971; Caldwell, D.R. et al, 1968). In order to measure the effect of carbon dioxide on the yield of Sb1, growth yield was measured with quantitative additions of carbon dioxide in the form of sodium bicarbonate. Standard medium without glucose was equilibrated to 39 deg. C. under O_2-free N_2 and 50 ml quantities were added to 140 ml nephelometer flasks. Sodium bicarbonate was added by syringe from roll tubes containing sterilized 555 mM/L $NaHCO_3$, kept cool to insure maximum solution of carbon dioxide.

In order to assure that no CO_2 was transmitted in the inoculum, the cultures were washed with CO_2-free medium by the following procedure. Overnight cultures were pressurized with 10 ml O_2-free N_2 and centrifuged upside down (rubber stopper down) in a Sorval bench centrifuge for 15 to 20 minutes. Supernatant was removed by inserting the needle of a 10 ml plastic disposable syringe through the rubber stopper opposite the side containing cells. Fresh CO_2-free N_2 equilibrated (without glucose) medium was transferred with a glass syringe to resuspend the pellet. It was necessary to use a glass syringe to transfer medium anaerobically because plastic syringes always gave oxidation of the medium transferred. The cells were washed twice in this manner resuspended in 4.0 ml CO_2-free medium and used for inoculation.

The results of this experiment are shown in table 2. The Yg estimate when carbon dioxide is absent is much lower than in the presence of carbon

Table 2: Growth of Sb1 with Carbon Dioxide at 3 mM PO_4

CO_2 (mM/1)	final pH	final glucose (mM/1)	dry wt. mg/1	Yg g/M
00	4.3	1.26	71.5	16.7
2.775	5.0	0.60	268	54.1
5.55	5.1	0	332	59.8
11.10	6.0	0	334	60.1

50 ml cultures were inoculated with 0.5 ml washed overnight culture of Sb1. Cultures contained 5.55 mM/1 glucose. CO_2 was added as a solution of $NaHCO_3$.

Table 3: Growth of Sb1 with Carbon Dioxide at 25 mM PO_4

CO_2 (mM/1)	final pH	final glucose (mM/1)	dry wt. (mg/1)	Yg (g/m)	mu (hr^{-1})	lactate (mM/1)
0.0	6.2	0	91.5	16.5	0.195	11.30
1.11	6.3	0	200	36.0	0.505	11.05
2.775	6.5	0	252	45.4	0.962	9.50
5.55	6.6	0	278	50.0	0.962	10.80
22.2	6.7	0	258	46.5	0.962	10.45
33.3	6.7	0	274	49.4	0.962	11.50

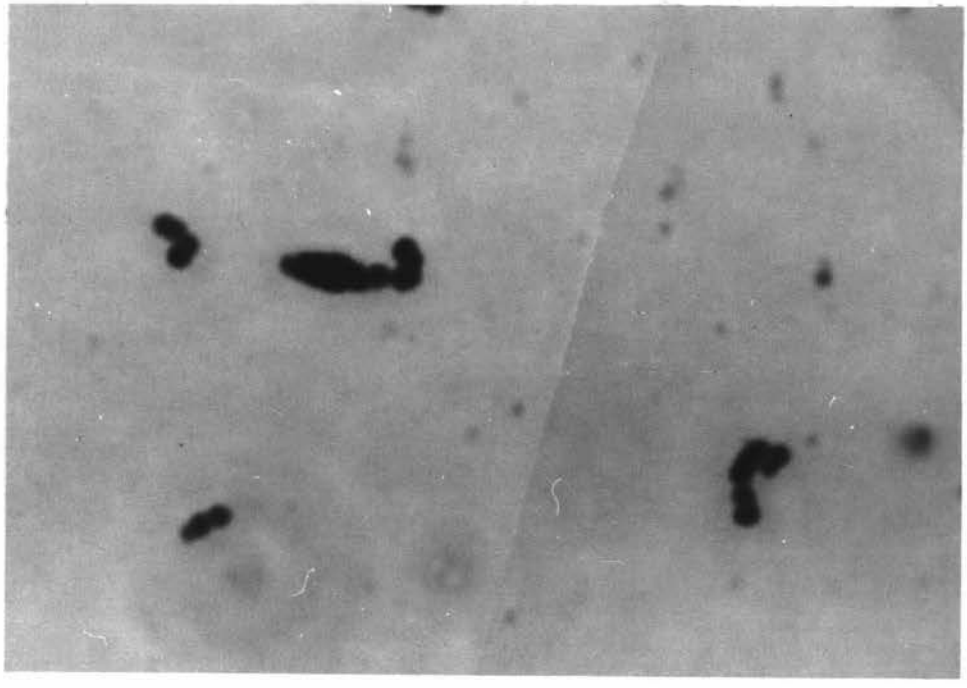
50 ml cultures were inoculated with 0.5 ml of washed overnight culture of Sb1. All cultures contained 5.55 mM/1 glucose. CO_2 was added as a solution of $NaHCO_3$.

Plate 3: Sb1 Grown Without CO_2

Plate 3 is a phase contrast photomicrograph of a batch culture of Sb1 grown in the absence of CO_2. Magnification ca x 2000.

Plate 4: Sb1 Grown With CO_2

Plate 4 is a phase contrast photomicrograph of a batch culture of Sb1 grown in the presence of CO_2. Magnification ca x 2000.



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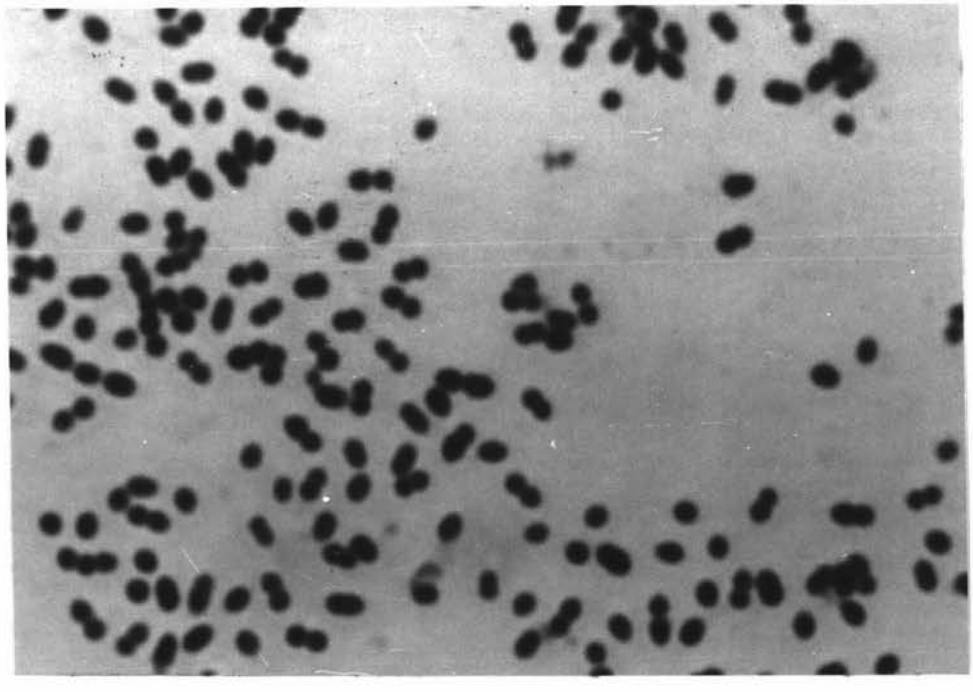


4

dioxide. This result is uncertain because the final pH is low, and there is remaining glucose. Therefore, the reduction in pH, due to the loss of buffering from carbonate, may have affected the growth of Sb1.

For this reason, the medium buffering was increased. It was found that the medium formed a precipitate at phosphate concentrations of 100 mM/1 but remained clear up to 50 mM/1 phosphate.

An experiment identical with the above, except for the presence in the medium of 25 mM/1 phosphate, gave the results shown in table 3. Under these conditions, the Yg in the absence of carbon dioxide is the same as at low phosphate. All glucose was consumed in the absence of carbon dioxide, indicating the prior low pH did not affect the cell yield. Growth yield becomes maximal at a carbon dioxide level of 5.55 mM/L, equivalent to the glucose concentration. Lactic acid production is constant at all levels of carbon dioxide. In the absence of CO_2, odd cell morphologies appear (plate 3) as opposed to those when CO_2 is present (plate 4).

III.1.1.5. Retention of 14-C labelled glucose and Carbon dioxide by Sb1.

Glucose retention was measured by measuring the fraction of the total culture activity retained by the cells at the end of logarithmic growth.

To test the possibility that the label retained by the cells was in the form of a reserve energy source, cells were collected at the end of logarithmic growth and again twenty four hours later. At the end of log phase growth, Sb1 retained 6.95 % of the total label. Twenty four hours later, Sb1 retained 5.69 %. Parallel to this experiment, a larger volume batch culture was grown. Dry weight samples were collected simultaneously with labelled cells. Dry weight at the end of log growth was 325 mg/L. Twenty four hours later it was 290 mg/L, a decrease of 13 %.

Retention of glucose at different initial concentrations of glucose was measured. In this experiment, the counts were not corrected to

disintegrations per minute. The cultures were all carried out in 140 ml nephelometer flasks (50 ml culture volume), except that containing 0.01 % glucose. The 0.01 % glucose culture was carried out at a larger volume (120 ml culture) in order to allow a more accurate measure of dry weight. The results of this experiment are shown in table 4.

Results of an experiment measuring glucose and carbon dioxide retention are shown in table 5. Carbon dioxide and glucose are both at concentrations of 5.55 mM/L. The fraction of label retained, and hence moles of each substrate, are approximately equivalent. The medium was modified as in reduced carbon dioxide experiments (Sect. III.1.1.4.).

III.1.1.5.2.

Results of an experiment measuring carbon dioxide and glucose retention are shown in table 6. In this experiment, an attempt was made to account for total activity by summing the activities of atmosphere, cells and supernatant. The activity in the supernatant was subjected to thin layer chromatography as for volatile fatty acids in order to determine labelled products. The cells were subjected to acid hydrolysis and chromatographed for autoradiography to determine the nature of the cellular label.

The cultures were grown in 50 ml volumes in 140 ml nephelometer flasks under a nitrogen atmosphere. Carbon dioxide and glucose concentrations were both 5.55 mM/L. Phosphate buffering was at 25 mM/L. In table 6, the figures represent total culture 14-C disintegrations per minute in millions. Total counts of carbon dioxide labelled cultures are the sum of supernatant, atmosphere and cell fractions, and percent values of each fraction are percent of that sum. Percent values of fractions from glucose labelled cultures are per cent of the total counts. The recovery of total counts was generally good.

Table 4: ^{14}C-glucose retention at differing initial levels of glucose.

Glucose (mM/L)	total cpm/ml	cellular cpm/ml	dry wt. mg/L
0	70	6.1	0
0.55	7780	576 (9.0)	30
5.55	21500	1930 (8.6)	231
27.8	21200	1510 (6.1)	838

Unbracketed figures are uncorrected cpm/ml.

Bracketed figures are % of total.

Table 5: ^{14}C Labelled Glucose and CO_2 Retention by Sb1.

14-C label	total dpm/ml	cellular dpm/ml	gas dpm	% label retention
CO_2	105310	10000	91909	9.5
Gluc.	455559	40000	556	8.7

Culture contained 5.55 mM/L CO_2 and glucose.

Table 6: ^{14}C Labelled Glucose and CO_2 Retention by Sb1.

14-C label	CO_2	total dpm/	spnt dpm	gas dpm	cellular dpm	% recovery
CO_2*	+	___	15.70	9.39	0.016	___
gluc.*	+	25.05	24.87	0.04	0.214	100
CO_2	+	___	3.33	15.00	1.13	___
gluc.	-	25.16	23.50	0.13	1.88	101
gluc.	+	25.01	21.95	2.49	3.13	110

Figures are total dpm per flask in millions.

Starred labels are uninoculated cultures.

Table 7: TLC of Steam Distillate of Culture Supernatant of Sb1.

14-C label	CO_2	Total dpm(1)	mEq acid	origin dpm	acetate dpm	formate dpm	% recovery
CO_2*	+	0.079	0.0	0.011	0.008	-	24
Gluc.*	+	0.54	0.0	0.022	0.038	-	111
CO_2	+	0.094	4.38	0.0	0.019	0.028	50
Gluc.	-	4.40	3.34	0.312	0.995	0.236	35
Gluc.	+	4.88	4.26	0.145	1.191	0.425	36

Starred labels are uninoculated samples. Radioactivities are expressed as dpm per flask in millions.

The fraction of label retained by cells is comparable to prior experiments.. The fraction of label in the atmosphere in 14-C carbon dioxide labelled cultures is much greater (1.6 x) in the inoculated culture than the blank. This is most probably due to the acid production by Sbl and consequent shift in solubility equilibrium of carbon dioxide. In the 14-C glucose labelled cultures, it is noteworthy that the amount of label in the atmosphere of the culture containing carbon dioxide is twenty fold that in the culture lacking carbon dioxide. This would imply an exchange between metabolic product of glucose and free carbon dioxide.

As shown in table 7, there is a significant amount of steam volatile acid produced in Sbl cultures. In order to determine the labelling in individual acids, these steam distillates were chromatographed and the individual acid spots eluted and counted. The total dpm in millions per flask for each spot are shown in table 7. From these results, it can be seen that in carbon dioxide labelled cultures, little activity is incorporated into steam volatile products. In glucose labelled cultures, approximately 18 % of the label is recovered in steam volatile products. While recovery of activity from chromatograms only approaches 50 %, the pattern of labelling remains noteworthy. The majority of the label appears in acetate (4.0 - 4.8 %), while a significant amount appears at the origin (0.6 - 1.2 %) as well as in formate (0.9 - 1.7 %).

The distribution of label from a chromatogram of culture supernatant is shown in table 8. In this case, the total recovery of label from the chromatogram is approximately 50 %. The high label in the acetate band of the glucose blank is due to smearing of glucose from the origin and encompassing the acetate spot. This would not affect other results, as glucose has been consumed in inoculated cultures. In the 14-C carbon dioxide labelled culture, the majority of the label remained at the origin, but a significant amount (0.6 % of the total) appears in formate.

Table 8: TLC of Supernatant of ^{14}C-Labelled Cultures of Sb1.

14-C label	CO_2	total dpm	origin dpm	lactate dpm	acetate dpm	formate dpm	% recovery
CO_2*	+	15.70	0.002	___	0.036	-	0.242
Gluc.*	+	24.87	6.576	-	5.338	-	48.0
CO_2	+	3.33	0.355	0.032	0.037	0.119	16.3
Gluc.	-	23.50	0.687	7.124	1.537	0.606	42.4
Gluc.	+	21.95	0.741	10.96	1.470	0.807	63.6

Figures represent total dpm per flask in millions.

Starred labels are for uninoculated cultures.

The percent label of the two 14-C glucose labelled cultures is nearly identical: 2.9 % at the origin, 36 % in lactate, 6 % in acetate, and 2.8 % in formate.

The distribution of label from an autoradiograph of the acid hydrolysate of the 14-C glucose labelled culture containing carbon dioxide is shown in table 9. All of the heavily labelled spots are identical with ninhydrin positive spots on the plates, indicating that the major retention of label is in amino acids. A faint label near the origin (probably a sugar phosphate) is insignificant. The labelling pattern from the glucose without carbon dioxide culture gives identical results.

Table 9: Autoradiogram of Sb1 Labelled with ^{14}C-Glucose

Spot	R_1	R_2	ninhydrin reaction
1	0.314	0.325	+
2	0.348	0.398	+
3	0.440	0.448	+
4	0.491	0.441	+
5	0.560	0.446	+
6	0.624	0.558	+

The above results are from the culture grown in the presence of 5.55 mM/1 CO_2. The results listed below are from the culture grown in the absence of CO_2.

Spot	R_1	R_2	ninhydrin reaction
1	0.238	0.483	+
2	0.419	0.595	+
3	0.481	0.615	+
4	0.555	0.475	+

III.1.2. Batch Culture of WV1

III.1.2.1. Cultural Characteristics of WV1

WV1 is a gram negative slightly curved rod in fresh batch liquid cultures. The cells frequently appear in pairs, but may be chained. The number of cells in a chain is difficult to distinguish. Cellular diameter is consistently 0.5 to 0.7 micro-meters. Cell length may vary from 2.0 micro-meters, in a young batch culture, to occassional cells 40 micro-meters in length in older cultures.

Colonial morphology in glucose agar roll tubes is shown in plates 5, 6, 7. Subsurface colonies are multiple lobed, generally showing three, frequently more (plate 5) disc shaped lobes. Surface colonies may either be circular with an entire edge (plate 6) or rhizoid (plate 7). Colonies are visible after 24 hours incubation at 39 degrees C., but 48 hours of incubation are necessary to give a countable colony size.

Growth on a variety of substrates is shown in table 1. The same conditions apply as for Sb1.

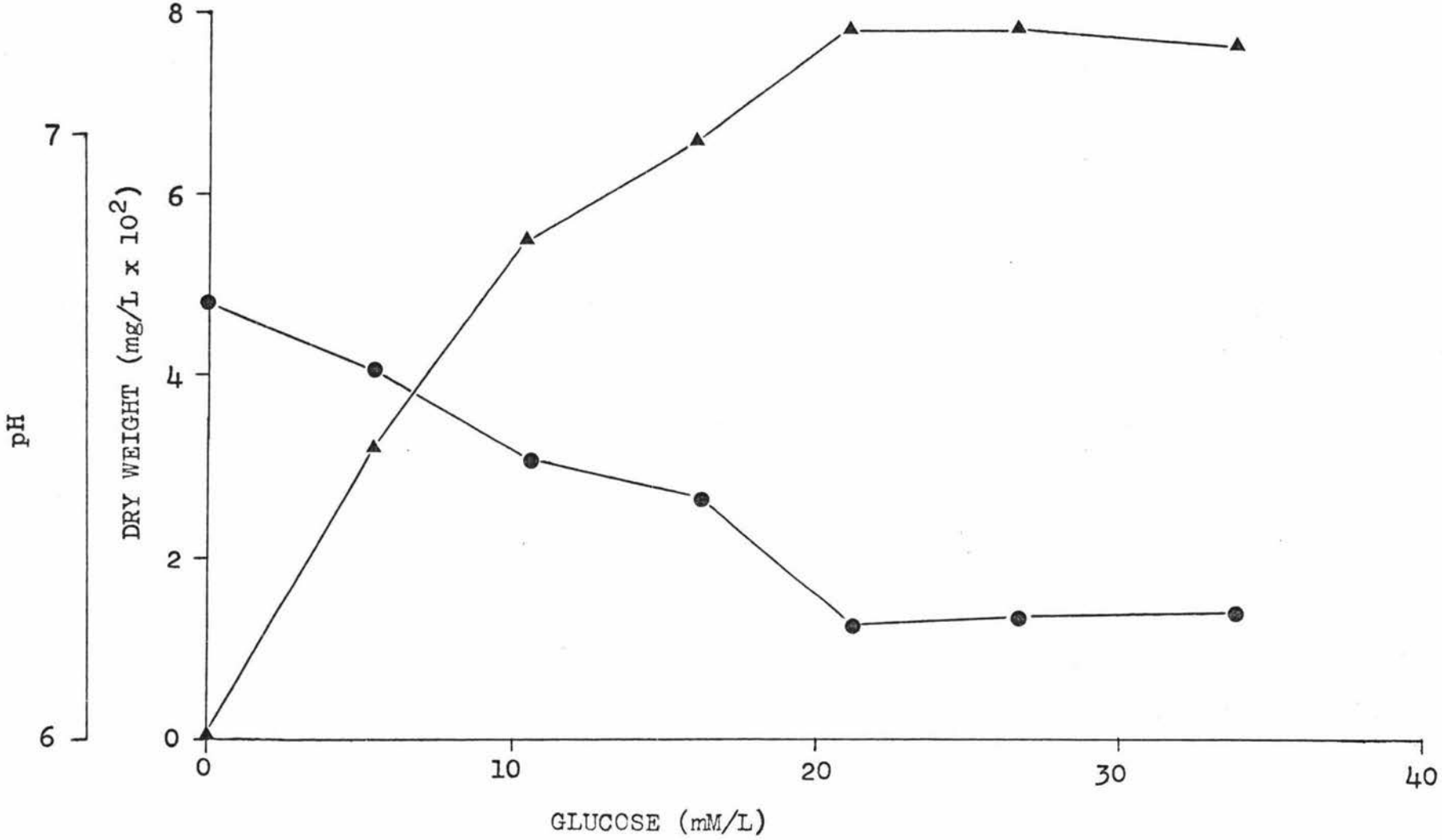
The same methods were used in efforts to demonstrate reserve material in WV1 as in Sb1 (Section III.1.1.1.) with the same negative results. In the demonstration of capsular material in Sb1 (phase examination of an india ink wet mount) WV1 was only barely distinguishable from the ink granules.

III.1.2.2. Growth Yield of WV1 with Glucose

The growth yield of WV1 with glucose is shown in figure 9. Over the range of zero to twenty milli-moles glucose per liter, glucose is growth limiting. Above twenty milli-moles per liter, there is no increase in dry weight of WV1. The slope of the line (Yg) from zero to ten milli-moles per liter is 55. The final pH drops linearly with increasing glucose concentration from pH 6.7 to pH 6.2 over the range zero to twenty milli-moles glucose per liter. The plateau in pH parallels the plateau in dry weight.

Figure 9: Dry Weight Yield of WV1 with Glucose
Cultures were grown in 50 ml cultures on standard medium with specific concentration of glucose as shown. Cells were collected at the beginning of stationary phase.

▲ , dry weight; ● ,final pH.


8
7
6
4
2
0
pH
DRY WEIGHT (mg/L x 10^2)
0
10
20
30
40
GLUCOSE (mM/L)

III.1.2.3. Growth Yield of WV1 with Trypticase

In order to observe the effect of trypticase and yeast extract concentration on growth yield of WV1, batch cultures were grown in standard medium at 5.55 mM/L glucose with varying levels of trypticase and yeast extract. The medium was prepared in the same manner as in section III.1.1.3. The results of this experiment are shown in figure 10.

Specific growth rate is maximal at 10 grams trypticase per liter. A Lineweaver-Burke plot of equation 2, where trypticase is S, gives a Ks of 0.378 gram trypticase per liter.

Above 2 grams trypticase per liter the dry weight increase is approximately linear and has an extrapolated intercept of approximately 380 mg per liter at zero trypticase concentration. This gives a Yg estimation of approximately 68 grams per mole at zero trypticase concentration.

III.1.2.4. Growth Yield of WV1 with CO_2

Growth yield of WV1 with CO_2 was measured for the same reasons as Sb1 (Sect. III.1.1.4.). Medium and inoculum were prepared in the same manner as previously descrided (Sect. III.1.1.4.). The results of an experiment with poorly buffered medium (3 mM/L phosphate) are shown in table 10. Results at carbon dioxide levels above zero are obscured due to the large reduction in pH, which is partially overcome at a bicarbonate concentration of 11.1 mM/L. It is clear from the zero level of CO_2 that CO_2 is required for the growth of WV1.

Results from an experiment in which phosphate buffering was increased to 25 mM/L are shown in table 11. In this experiment, the pH is reduced no more than 0.4. Glucose is entirely consumed at a level of CO_2 of 2.77 mM/L, but yield and specific growth rate do not reach a maximum until a concentration of CO_2 of 22.2 mM/L is reached. Cellular morphology at low CO_2

Figure 10: Growth Yield of WV1 with Trypticase

Cultures were grown in 50 ml volumes on standard medium at 5.55 mM/L glucose with specific additions of trypticase as shown. Cells were collected at the beginning of stationary phase.

▲ , dry weight; ● , specific growth rate.

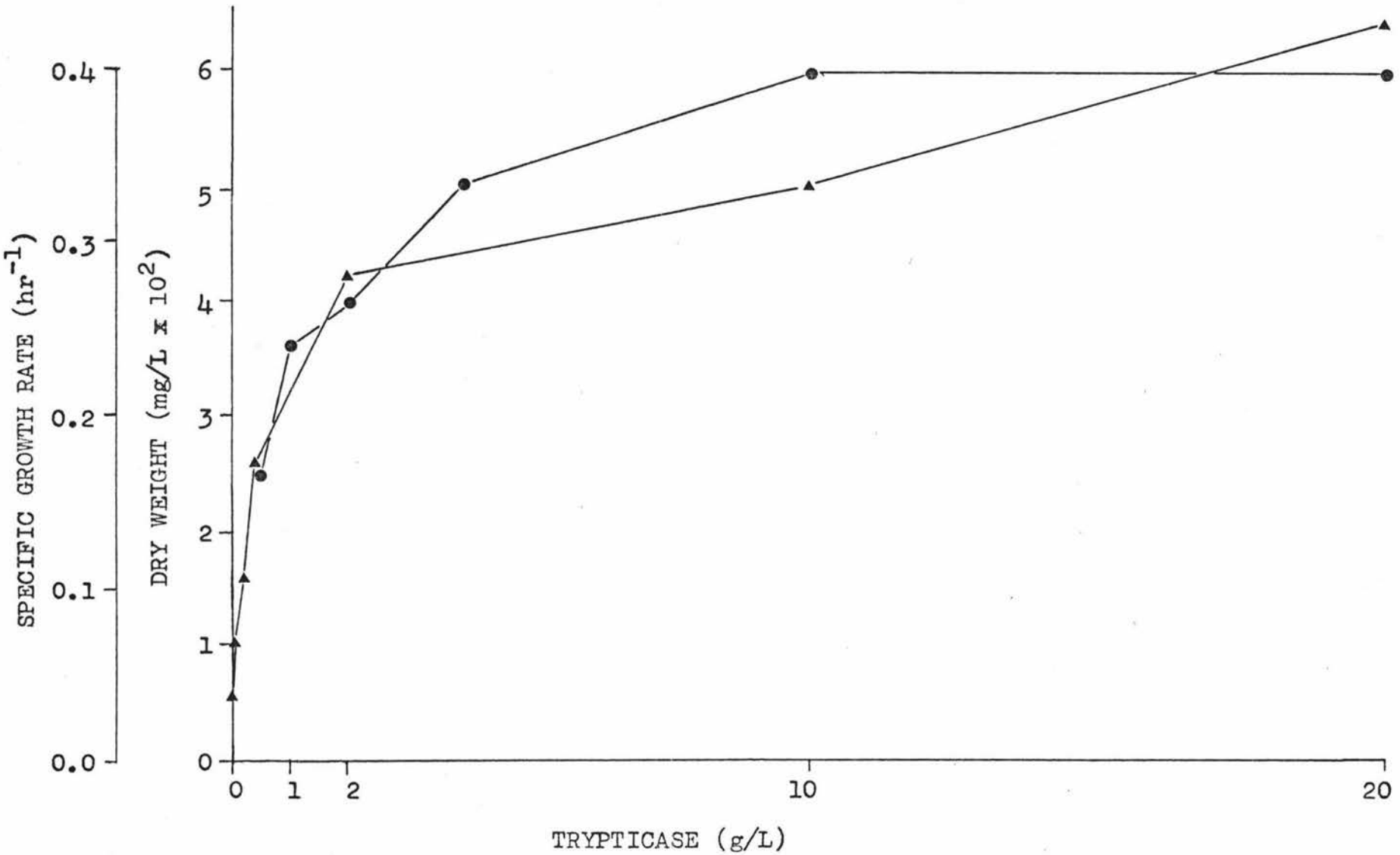

SPECIFIC GROWTH RATE (hr^{-1})
0.0
0.1
0.2
0.3
0.4
DRY WEIGHT (mg/L x 10^2)
0
1
2
3
4
5
6
0
1
2
10
20
TRYPTICASE (g/L)

Table 10: Growth of WV1 with CO_2 at 3 mM PO_4

CO_2 mM/L	final pH	final glucose mM/L	dry wt. mg/L	Yg g/m
0	6.2	6.04	20	3.3
2.78	4.9	0.805	107	20.6
5.55	4.9	0.033	143	23.8
11.1	5.7	0.0	208	34.7

Cultures were grown with standard medium less carbonate under nitrogen gas. The phosphate buffer was at 3 mM/L.

Table 11: Growth of WV1 with CO_2 at 25 mM PO_4

CO_2 mM/L	final pH	final glucose mM/L	dry wt. mg/L	Yg g/m	mu hr^{-1}	Y_{CO_2} g/m
0.0	6.8	4.84	5.7	0.8	-	-
1.11	6.7	3.78	51.5	29.1	-	41.2
2.78	6.4	0.0	191.5	34.5	0.184	67.0
5.55	6.5	0.0	260	46.9	0.231	45.8
22.2	6.8	0.0	318	57.3	0.357	14.1
33.3	6.8	0.0	326	58.8	0.357	9.6

Cultures were grown in standard medium less carbonate under nitrogen gas. The phosphate buffer was at 25 mM/L.

concentration (1.11 mM/L) is altered (plate 8) from that at higher CO_2 concentration (5.55 mM/L; plate 9).

III.1.2.5. Retention of 14-C labelled glucose and CO_2 by WV1.

An experiment identical with that performed on Sb1 (Section III.1.1.5.) to measure the fraction of glucose retained at the end of logarithmic growth and the decrease in label during 24 hours of stationary phase was performed on WV1. At the end of log phase, the cells contained 14.5 % of the label. Twenty four hours later the fraction had decreased to 10.1 % of the total. Over the same time, dry weight of the cells decreased from 295 mg/L to 257 mg/L, a decrease of 11 %.

An experiment measuring the retention of label over a range of glucose concentrations was performed. From substrate concentrations of 0.555 and 5.55 mM/L, the per cent retention of label were 7.1 and 9.0, respectively.

Since carbon dioxide was known to be a requirement for growth of WV1, it was decided to measure the fraction of CO_2 incorporated into cell material. At the same time, glucose label retention was also measured. The medium containing 5.55 mM/L CO_2 and glucose under nitrogen and the medium was buffered with 25 mM/L phosphate. 11.9 % of the CO_2 label and 10.4 % of the glucose label was retained by the cells. A count of a sample of the gas phase gave a high activity from the labelled glucose culture. This was a qualitative result, as the actual fraction of the gas phase sample was not known.

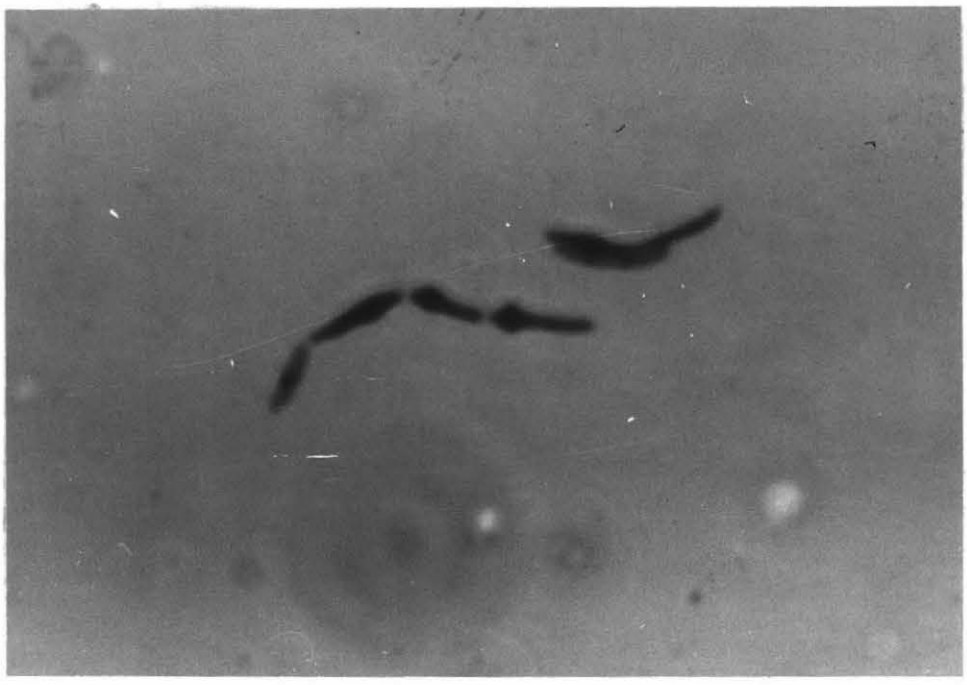
A final experiment was performed on WV1 to measure the retention of 14-C labelled CO_2 and glucose and account for the label in the three fractions: cells, supernatant and gas phase. The culture conditions were as above and the inoculum was washed as described for Sb1 (III.1.1.4.). The results of this experiment are shown in table 12. Due to loss of total

Plate 8: WV1 Grown on Low CO_2

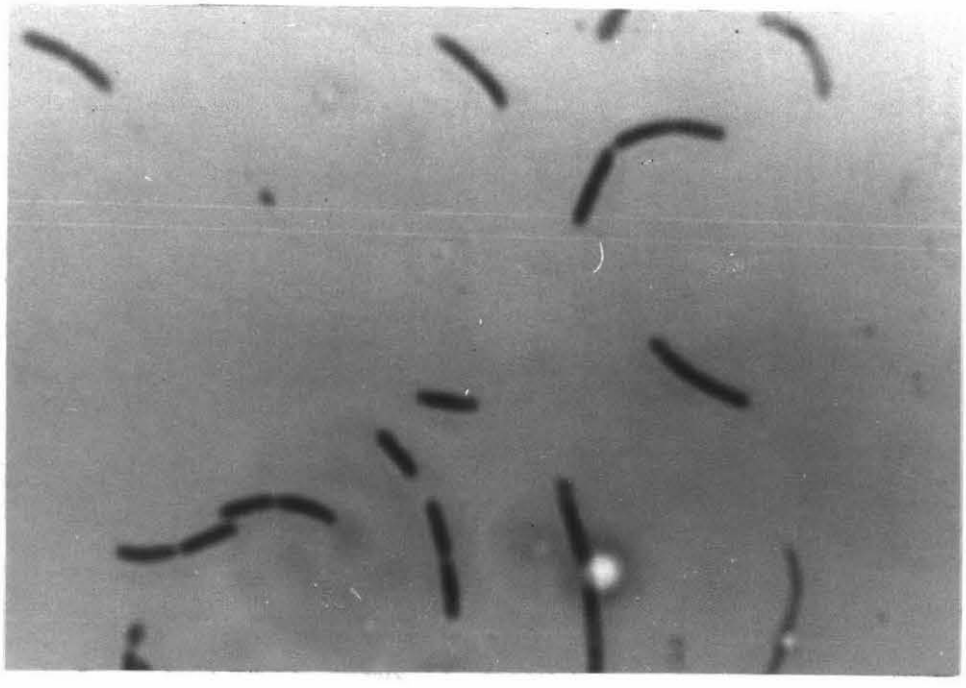
Plate 8 is a phase contrast photomicrograph of batch culture of WV1 grown at 1.11 mM/1 $NaHCO_3$.
Magnification ca x 3000.

Plate 9: WV1 Grown on High CO_2

Plate 9 is a phase contrast photomicrograph of a batch culture of WV1 grown on 5.55 mM/1 $NaHCO_3$.
Magnification ca x 3000.



8



9

Table 12: Retention of ^{14}C-Labelled CO_2 and Glucose by WV1.

14-C label	total dpm	spnt dpm	gas dpm	cells dpm	% recovery
*CO_2	-	15.70 (62.5)	9.39 (37.4)	0.02 (0.06)	-
*glucose	25.05	24.87 (99.3)	0.04 (0.16)	0.21 (0.86)	101.3
CO_2	-	10.92 (49.0)	10.44 (46.7)	0.99 (3.90)	-
glucose	25.40	20.28 (80.0)	1.09 (4.29)	2.16 (8.50)	92.7

Figures represent the total dpm per flask in millions. Starred labels indicate uninoculated flasks. Bracketed figures are % of the total.

Table 13: TLC of Steam Distillate of Culture Supernatant of ^{14}C Labelled Cultures of WV1.

14-C label	acid mEq/l	steam volatile dpm	origin dpm	acetate dpm	formate dpm	butyrate dpm	% recovery
*CO_2	0	0.079	0.011	0.008	-	-	24
*glucose	0	0.054	0.022	0.038	-	-	111
CO_2	11.45	6.69	0.180	0.626	1.456	0.833	46
glucose	11.76	15.46	0.160	0.852	1.626	6.564	60

Figures represent total dpm per flask in millions. Starred labels are uninoculated flasks.

activity samples for 14-C CO_2 labelled flasks, the percent values reported are percent of the sum of the fractions. The total activity is near that expected from the amount initially added to each flask (expected total activity of 25 x 10^6 dpm/flask). The amount of label in the gas phase of 14-C CO_2 labelled inoculated culture differs insignificantly from that of the inoculated culture. The amount of 14-C CO_2 retained by cells is lower by approximately one half that found in previous experiments. The fraction of label retained by cells in labelled glucose culture is comparable with previous results.

In order to measure the amount of label in steam volatile products and to determine a ratio between them, the supernatant was steam distilled, the total acid measured, and the steam distillate was chromatographed on 1 mm plates for volatile fatty acids. The acid spots were removed and counted. The results of these measurements are shown in table 13. The low recovery of activity is probably due to the period of heating during development of the chromatograph. The amount of acid produced is of the order expected, approximately 2 equivalents per mole of glucose. The ratio of label in formate and butyrate from the labelled glucose culture is approximately 1:4, respectively. This implies an equimolar production of formate and butyrate. The high label in the formate band of the labelled CO_2 culture indicates a role for CO_2 as proton acceptor, or that there is an exchange between CO_2 and a predecessor of the fatty acid products. The amount of label recoverable as steam volatile products was 30 % and 61 % for ^{14}C labelled CO_2 and glucose cultures respectively.

The culture supernatant was chromatographed for volatile fatty acids and the spots were eluted and counted similarly to the steam volatile fraction. The results of these measures are shown in table 14. Approximately the same ratios resulted, although larger activities were recovered. The recovery of label was similar to that from chromatography of the

Table 14: TLC of Supernatant of 14-C Labelled Cultures of WV1.

14-C label	spnt. dpm	origin dpm	acetate dpm	formate dpm	butyrate dpm	spnt. % recovery	v.f.a. % recovery
*CO_2	15.70	0.002	0.036	-	-	0	-
*glucose	24.87	6.576	5.338	-	-	48	-
CO_2	10.92	1.486	0.654	3.335	0.743	57	93
glucose	20.28	2.005	1.128	2.737	8.014	68	90

Figures represent total dpm per flask in millions. Starred labels are uninoculated flasks.

volatile fraction when supernatant activity is the reference, but is considerably higher (90 %) if the activity of the steam volatile fraction is the reference.

Results of an autoradiogram of an acid hydrolysate of a 14-C glucose labelled culture of WV1 is shown in table 15. Three visibly labelled amino acids appeared in the hydrolysate (spots A, B, and C, table 15) and contained approximately one half of the total label recorded by the film (estimated by eye). Spot D was faint and fluoresced under ultraviolet light. Spots E F and G are not amino acid or nucleic acid. Subsequently to the ninhydrin spray, the plate was sprayed for carbohydrate (copper sulphate - phosphomolybdate p.243, Dawson, R.M.C. et al., 1969) and then for lipid (K Mn O_4, p.248, Dawson, R.M.C. et al., 1969). Neither of these sprays showed any reaction, but this could be due to interference from previous sprays.

Table 15: Autoradiogram of ^{14}C Glucose labelled WV1

14-C spot	R_1	R_2	ninhydrin reaction	UV fluor.	label intensity
A	0.34	0.40	+	-	10
B	0.44	0.45	+	-	1
C	0.50	0.46	+	-	3
D	0.65	0.62	-	+	1
E	0.77	0.56	-	-	3
F	0.80	0.64	-	-	8
G	0.81	0.67	-	-	7
H	0.83	0.78	-	-	1

III.2 Continuous Culture Results

III.2.1. Continuous Culture of Sb1

III.2.1.1. Initiation of Continuous Culture

All continuous cultures were begun by the following procedure. The growth vessel was flushed with a slow flow of oxygen free CO_2 for about 12 hours and then the pump was started and the growth vessel filled with medium. The medium was prepared as described (II.4.2.) to a glucose concentration of 5.55 mM/L. When the growth vessel was filled with medium, the rate of pumping was reduced to a low level, and the growth vessel was inoculated with an overnight culture of Sb1. When the culture had grown (four to six hours), the pump speed was increased, if necessary, to give the desired dilution rate.

III.2.1.2. Equilibration time of continuous cultures

A frequently used criterion for establishment of equilibrium is stabilization of optical density (Tempest, D. W. 1970; Dean, A. C. R., 1969). A measure more appropriate to continuous culture is the number of generations of the cell population, or the number of volume exchanges. A complete passive dilution of the growth vessel (no growth) of ten volume exchanges would give a concentration of the original reduced by a factor of 2^{-10} (or 0.1 % of the original concentration).

In order to estimate the number of generations necessary to give this equilibrium for Sb1, the optical density of the culture was observed after upward shifts in the rate of dilution. The experiment was repeated four days in succession with dilution rates of 2.22 hr^{-1}, 2.54 hr^{-1}, 2.80 hr^{-1} and 3.35 hr^{-1}. Between each period of increased rate (overnight) the dilution rate was returned to 0.54 hr^{-1} to preserve medium. At all rates

but the last, a stable optical density was reached in a smotthly decreasing curve within 10 volume exchanges. In the last case, it was reached after seven volume exchanges. Ten volume exchanges was accepted as the minimum to establish an equilibrium after a rate change. All reported equilibrium values are from samples taken after ten or more space volume exchanges.

Pigment production by Sbl was noticeably greater at some times than at others. Plate 10 shows the result of one experiment with Sbl at a moderate growth rate ($D = 2.0\ hr^{-1}$). This plate shows the layering of cells in the effluent reservoir. The bottom layer is medium pumped through prior to inoculation. The next layer is original growth. The deep red layer appeared just as growth reached maximum optical density after which the intensity of pigment began to decrease.

III.2.1.3. Equilibrium Results of Continuous Culture of Sbl

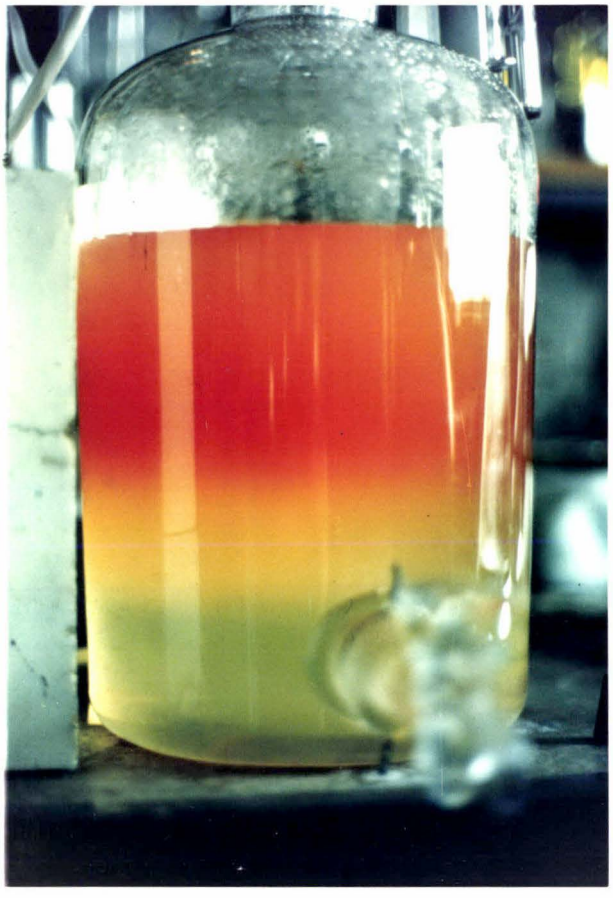
The results of determinations of colony forming units is shown in figure 11, and the results of total cell counts is shown in figure 12. The curve shows a steep increase from dilution rates of less than $0.1\ hr^{-1}$ to a rate of $0.3\ hr^{-1}$. At rates of $1.6\ hr^{-1}$ to rates of $3.0\ hr^{-1}$, the curves decrease with a positive curvature (as if approaching a positive asymptote). The overall ratio of total cell counts to CFU counts is 1.60 with a 95 % confidence limit of $\pm$ 0.18.

At low growth rates (less than $0.1\ hr^{-1}$) the number of determinations is too few (3) to give a good statistic. The mean ratio with 95 % confidence limits at low growth rates is 2.12 $\pm$ 1.17. The implication remains that there is an increased ratio at these rates, indicating a decreasing viability below dilution rates of $0.1\ hr^{-1}$.

The ratios of dry weight to total counts and to CFU counts and their 95 % confidence limits are 21.3 $\pm$3.6 and 25.9 $\pm$5.4. No significant

Plate 10: Continuous Culture of Sb1: Pigment Production

Plate 10 shows the sequence of pigment production in a continuous culture of Sb1. Culture effluent is layered from the bottom to the top of the reservoir.



10

Figure 11: Continuous Culture of Sb1: Summary of CFU with D

Figure 11 shows a summary of all equilibrium determinations of colony forming units. High points at D = 2.1 are anomalous.

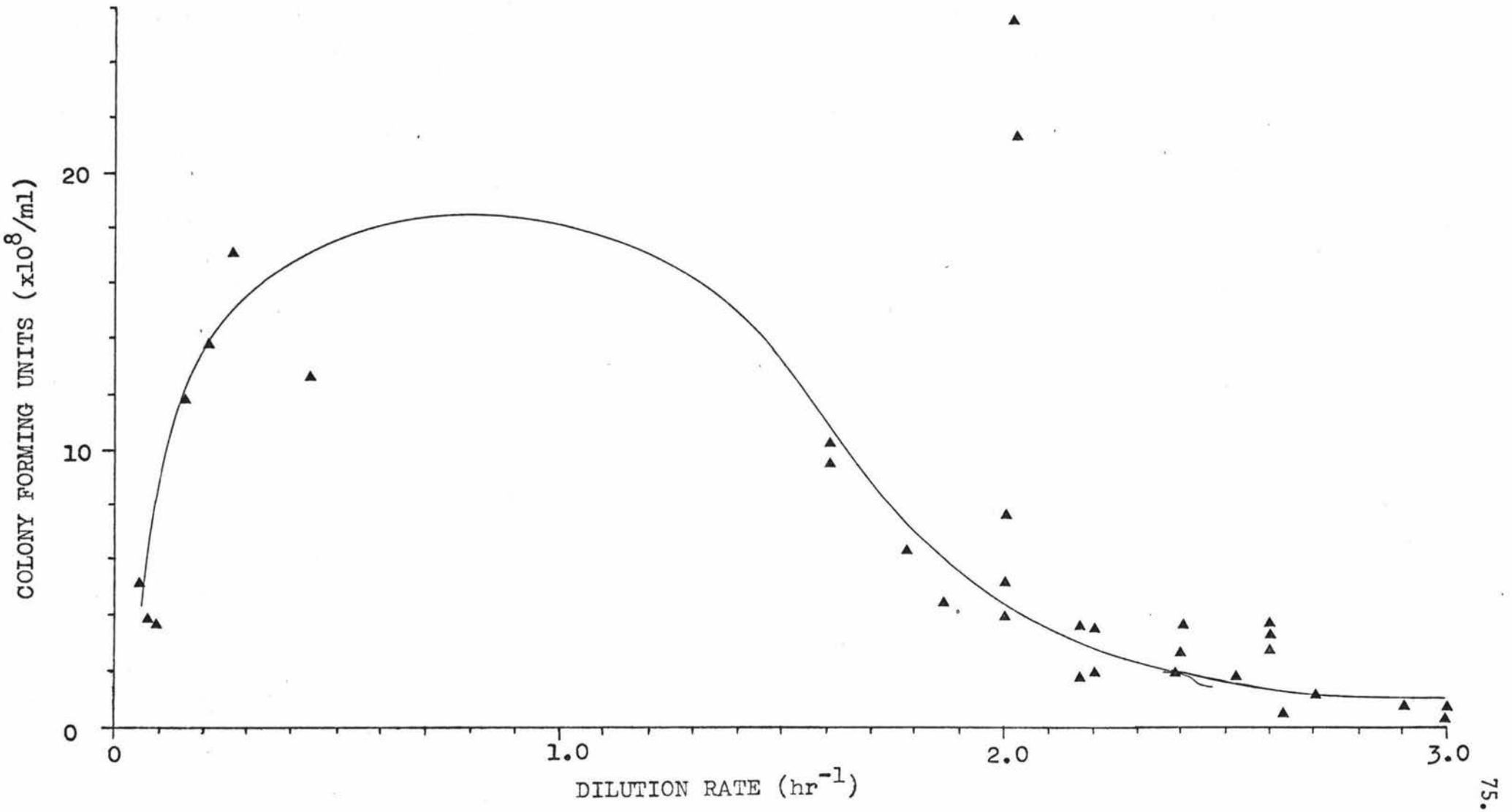
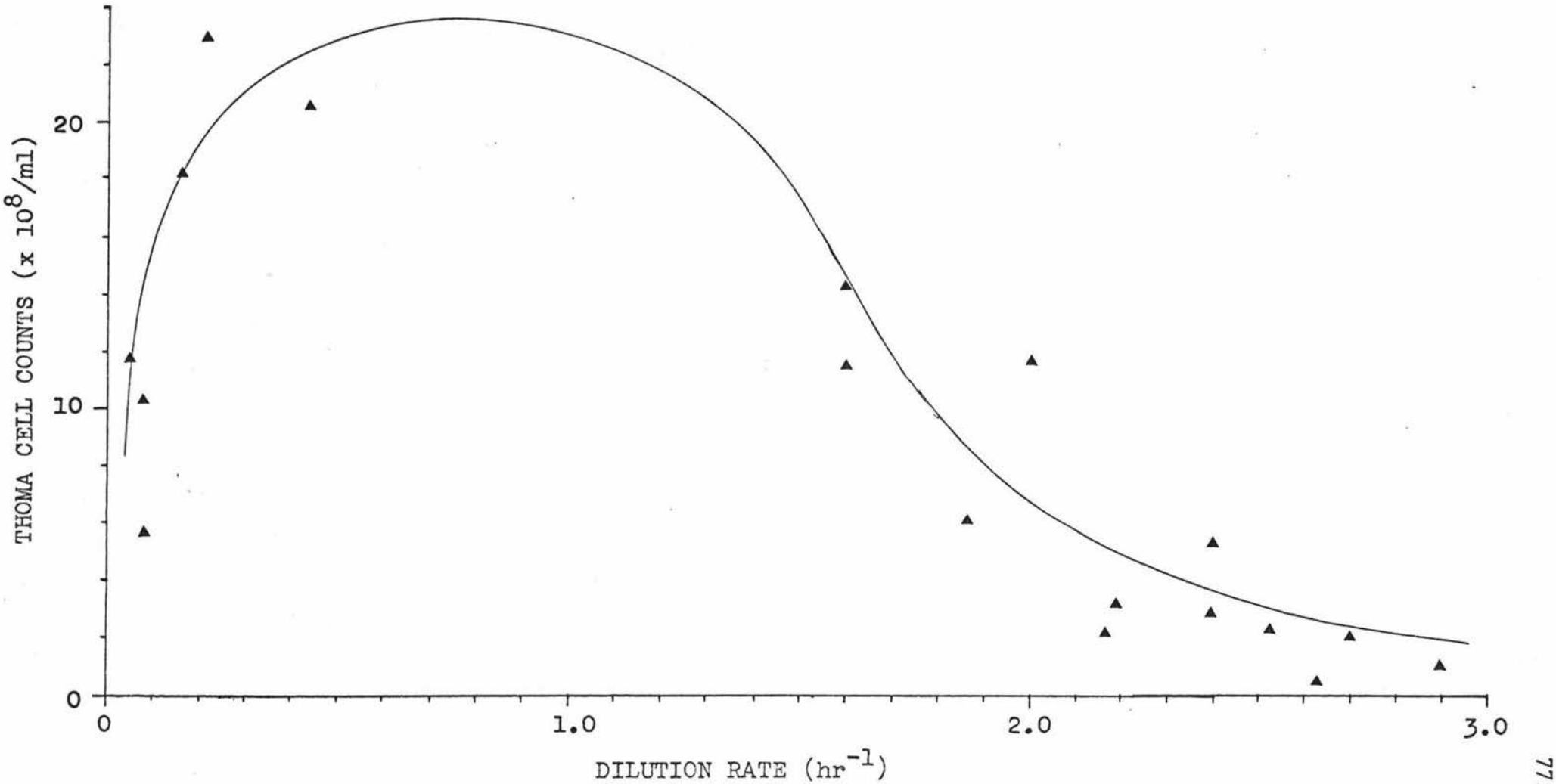

COLONY FORMING UNITS (x10^8/ml)
20
10
0
0
1.0
2.0
3.0
DILUTION RATE (hr^{-1})

Figure 12: Continuous Culture of Sb1: Summary of Total Cell Counts with D.

Figure 12 shows a summary of all Thoma cell counts at equilibrium conditions.


THOMA CELL COUNTS (x 10^8/ml)
20
10
0
0
1.0
2.0
3.0
DILUTION RATE (hr^{-1})

variation is noted at low or high growth rates.

III.2.1.4. Dry Weight Determinations at Equilibrium, Sb1

The results of dry weight determinations are summarized in figure 13. Dry weights are not significantly reduced at low growth rates. At high growth rates (greater than 1.8 hr^{-1}), dry weights decrease. The amount of scatter in the plot of dry weight makes interpretation difficult, but the curve appears to have positive curvature similar to that of the plot of CFU.

The average estimation of yields with 95 % confidence limits is 39.6 $\pm$ 7.6 grams per mole. There is no significant variation at low growth rates.

III.2.1.5. Estimation of Monod Growth Constants

If wall growth is present in continuous culture, there will be a deviation from the equation of Monod (equation 1) which is most significant at high growth rates. The deviation is manifested as a positive curvature at growth rates greater than the maximum specific growth rate (Topiwala & Hamer, 1971). For this reason, determinations of S and , taken at growth rates greater than 2.5. hr^{-1}, were omitted from the plot of 1/ vs 1/S shown in figure 14. A linear regression of the data forming this figure give the following equation:

$$1/\mu = 0.405 + 0.1738(1/S)$$

From this, μ_{max} is estimated as 2.47 hr^{-1} (td = 16.8 minutes) and Ks is 0.429 mM/l glucose.

III.2.2. Continuous Culture Results: WV1

III.2.2.1. Conditions for Growth

The Preparation of the growth vessel, medium, inoculum and criterion of equilibrium (at least ten volume exchanges) for continuous culture of

Figure 13: Continuous Culture of Sb1: Summary of Cell Yield

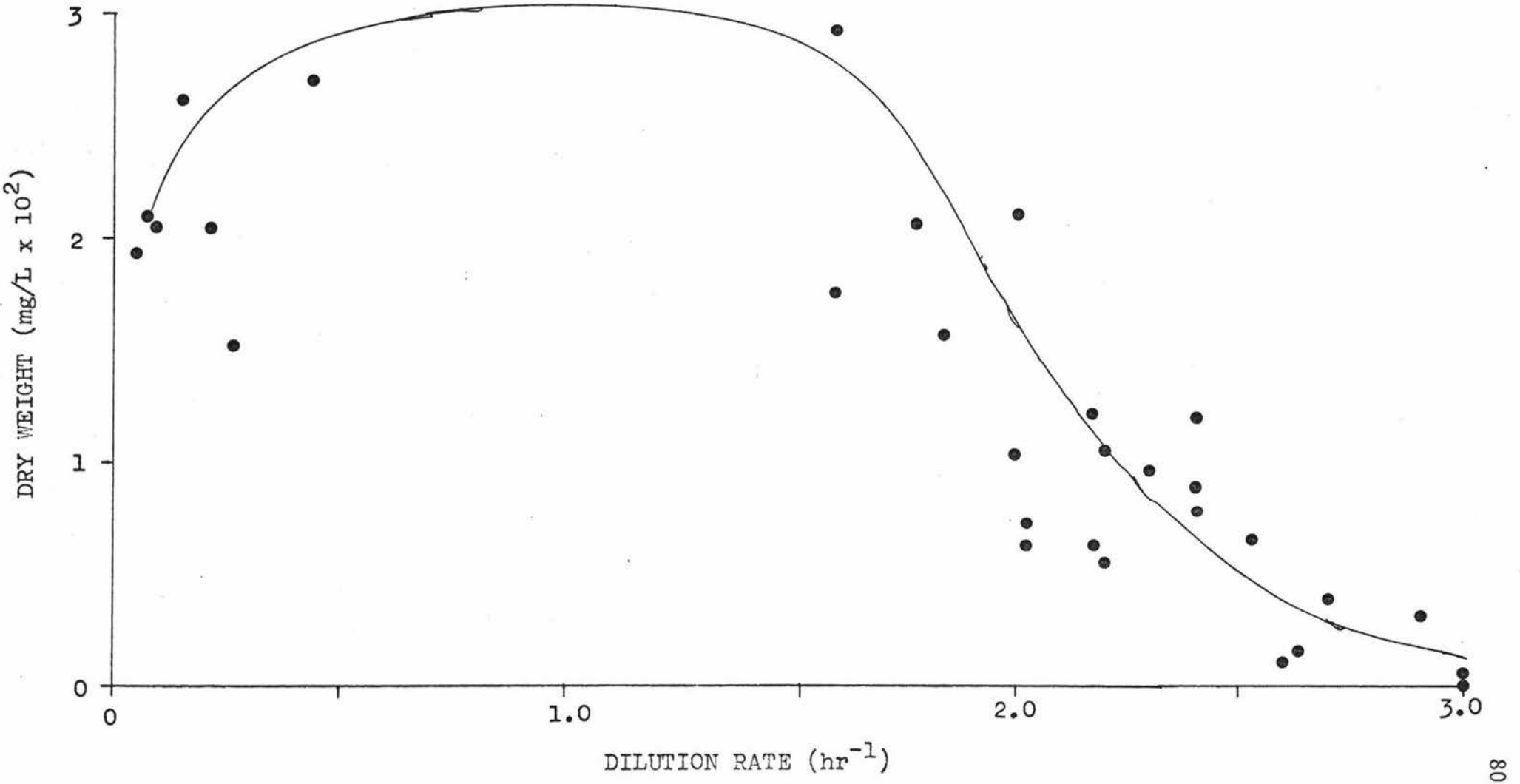
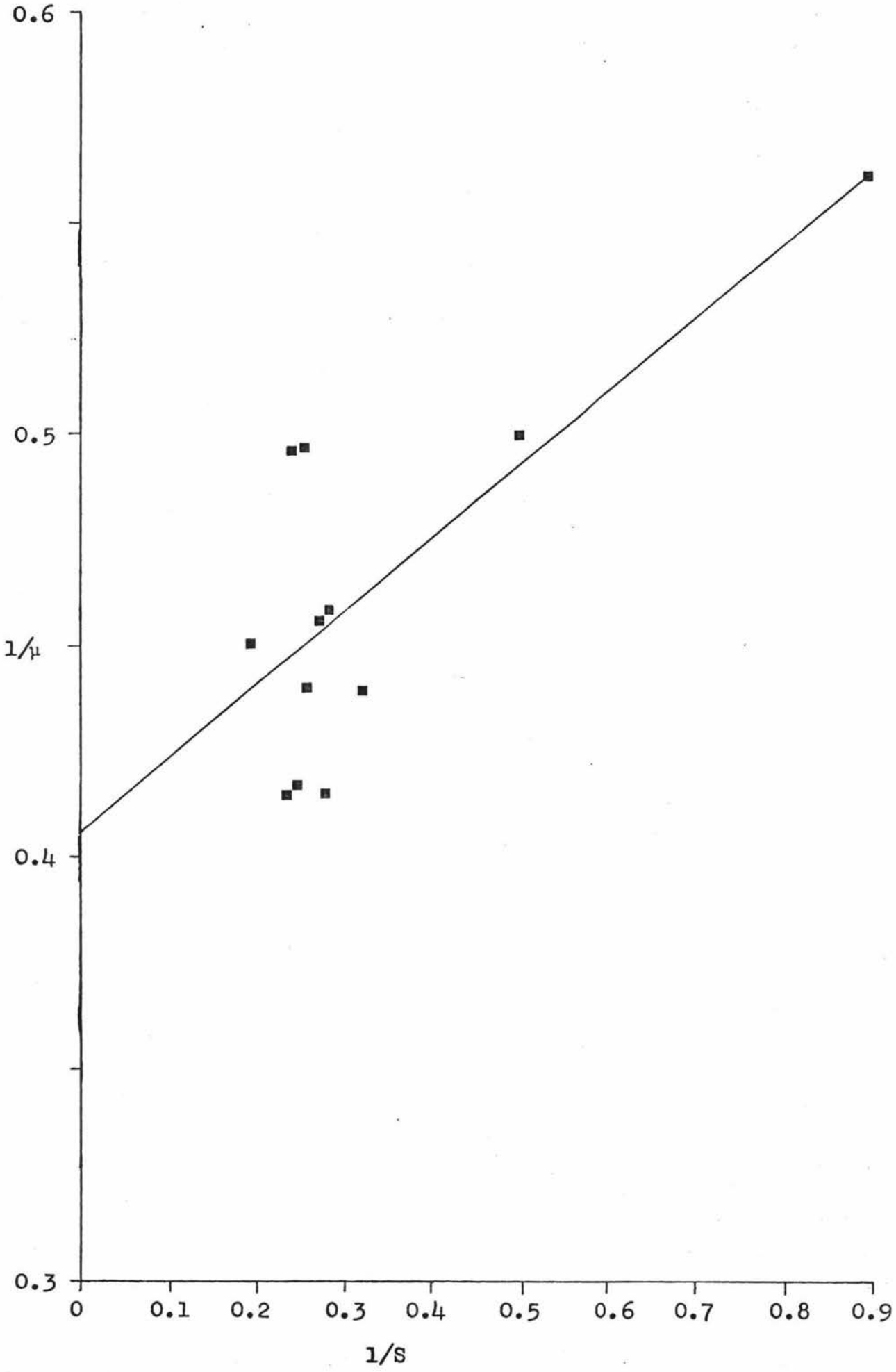

3
2
1
0
DRY WEIGHT (mg/L x 10^2)
0
1.0
2.0
3.0
DILUTION RATE (hr^{-1})

Figure 14: Continuous Culture of Sbl
Lineweaver Burke Plot

Figure 14 shows the Lineweaver-Burke plot of all equilibrium determinations of μ and S between D = 1.6 hr^{-1} and 2.5 hr^{-1}.


0.6
0.5
1/μ
0.4
0.3
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1/S

WV1 were the same as for Sb1 (Sect. III.2.2.2.&2.) except that a longer period of time was allowed for the culture to grow after inoculation before increasing the pumping rate.

A summary of determinations of total cells and CFU is shown in figure 15. Cell density is maximal at lower growth rates (0.05 hr^{-1}) and decrease gradually to near zero at a dilution rate of 0.7 hr^{-1}. The ratio of Thoma cell count to CFU and the 95% confidence limit is 3.42 ± 0.69. There is no significant deviation from this figure over the range of growth rates that were measured. The ratios of dry weight to total cell count and of dry weight to CFU and their 95% confidence limits are 113.1 ± 21.0 and 37.4 ± 8.1, respectively. There was no significant variation in either of these over the range of growth rates measured.

III.2.2.3. Dry Weight and Yield of WV1

Results of dry weight determinations are summarized in figure 16. The maximum production of cells is approximately 350 mg of cells per litre. Determinations of dry weight begin to decrease above growth rates of 0.5 hr^{-1} and approach zero at a specific growth rate of 0.7 hr^{-1}.

The average of all yield estimations and its 95% confidence limit is 69.1 ± 6.8 gram per mole of glucose. There is no significant variation from this overall average for any portion of the curve.

III.2.2.4. Estimation of the Monod Constants

A plot of equation (2) for all growth rates is shown in figure 17. There is no positive curvature of the dry weight of cell count plots or a plot of S (figure 18), so that all points above 0.3 hr^{-1} are included. The equation derived from a linear regression of these points is given below:

$$1 / \mu = 1.42 + 0.472(1 / S)$$

From this, $\mu_{max} = 0.704\ hr^{-1}$ (td = 59 min) and Ks = 0.332 mM / l.

Figure 15: Continuous Culture of WV1; Summary of CFU and Total Cell Counts

□, CFU; ■, Total Cell Counts

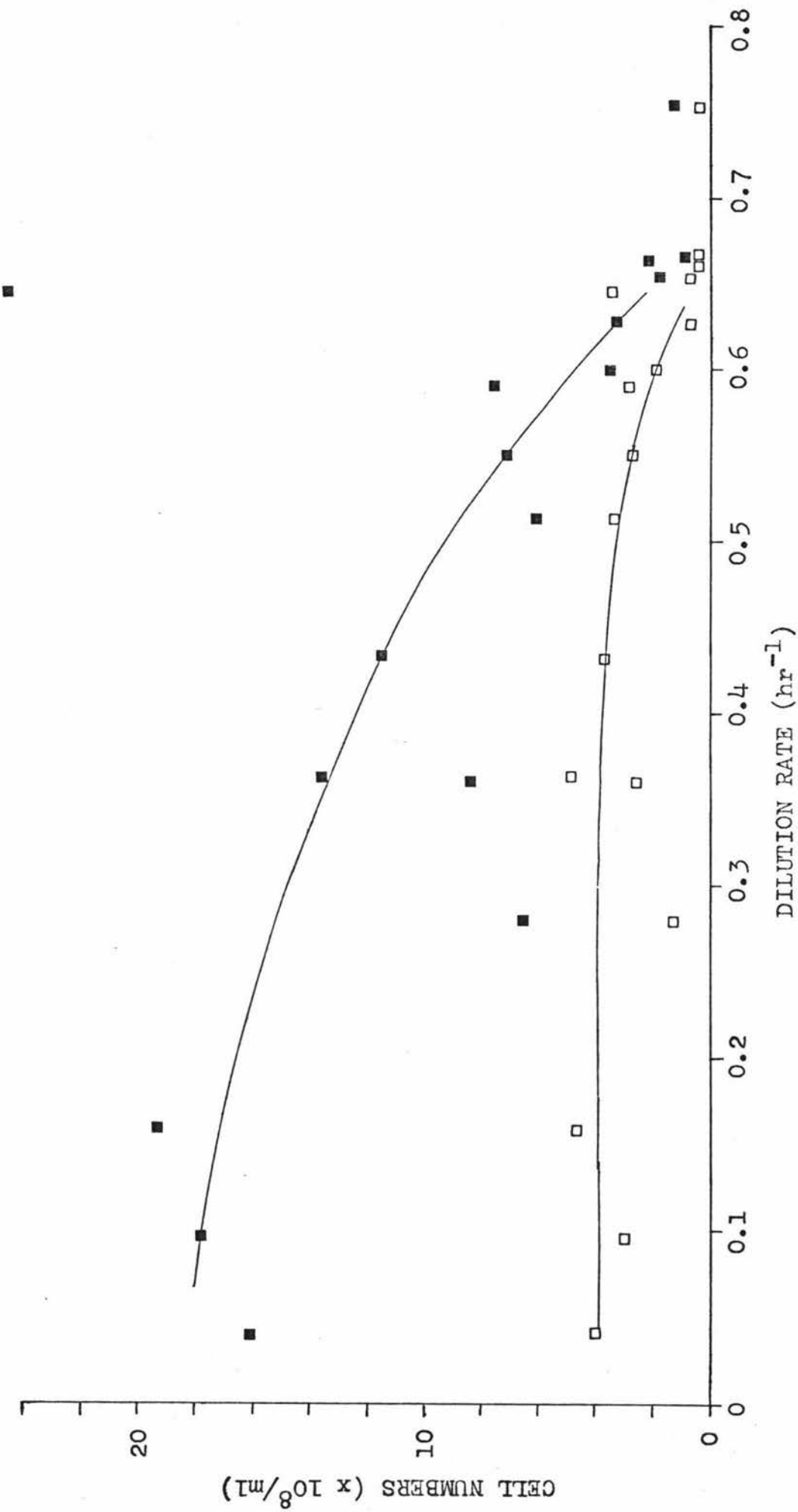

CELL NUMBERS (x 10^8/ml)
0
10
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
DILUTION RATE (hr^{-1})

Figure 16: Continuous Culture of WV1; Summary of Cell Yield

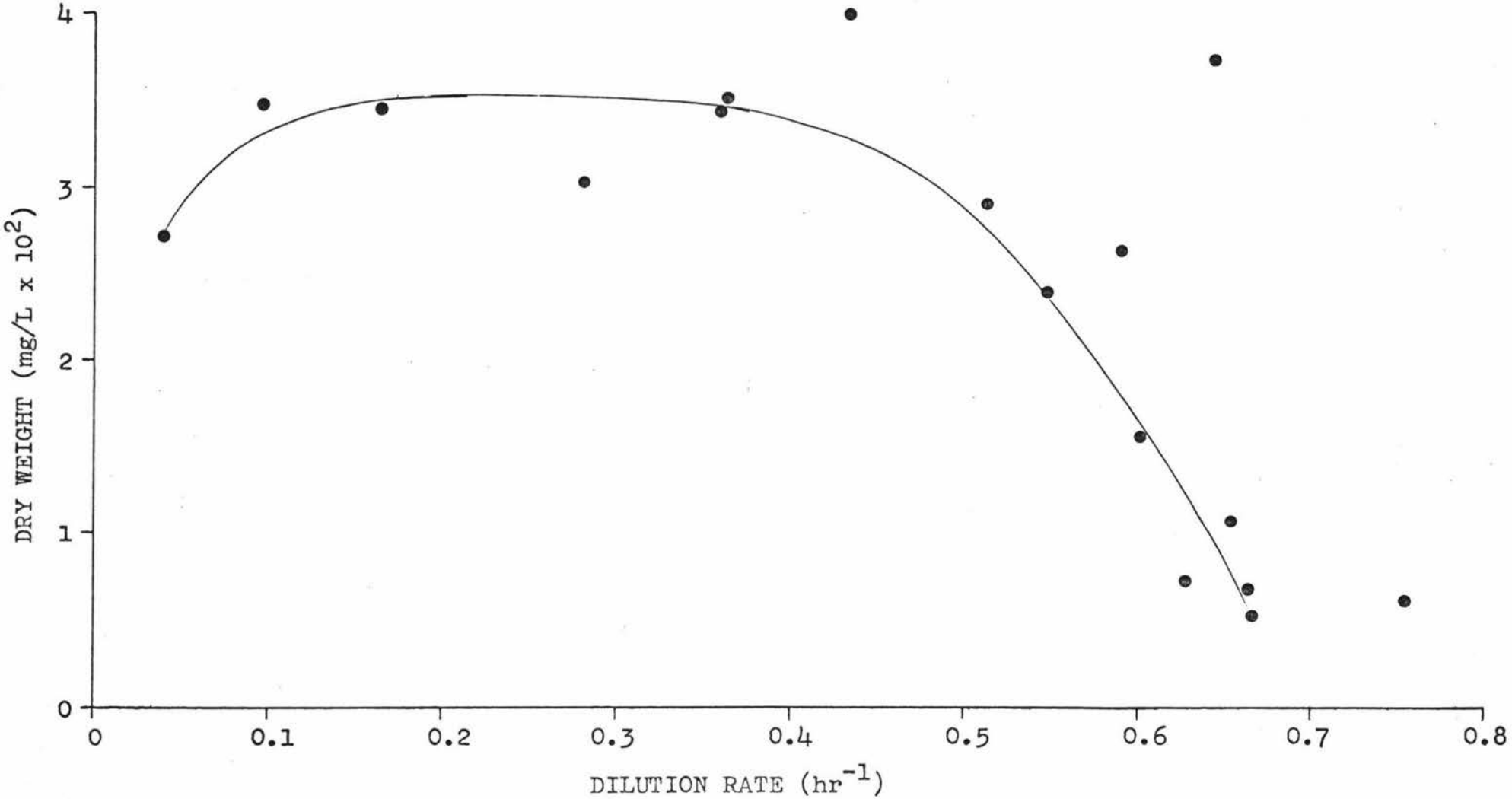

DRY WEIGHT (mg/L x 10^2)
4
3
2
1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
DILUTION RATE (hr^{-1})

Figure 17: Continuous Culture of WV1;
Lineweaver-Burke Plot

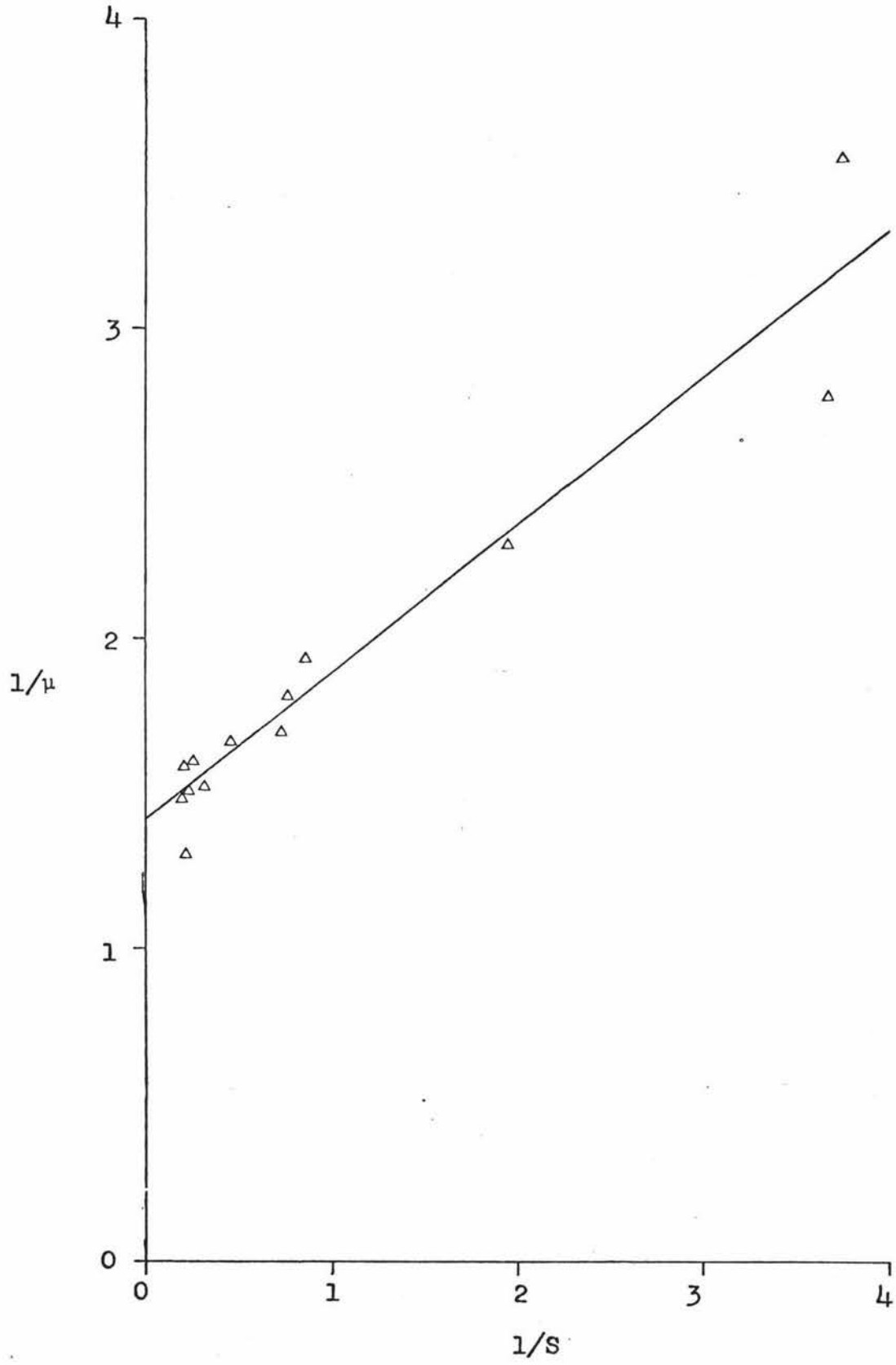
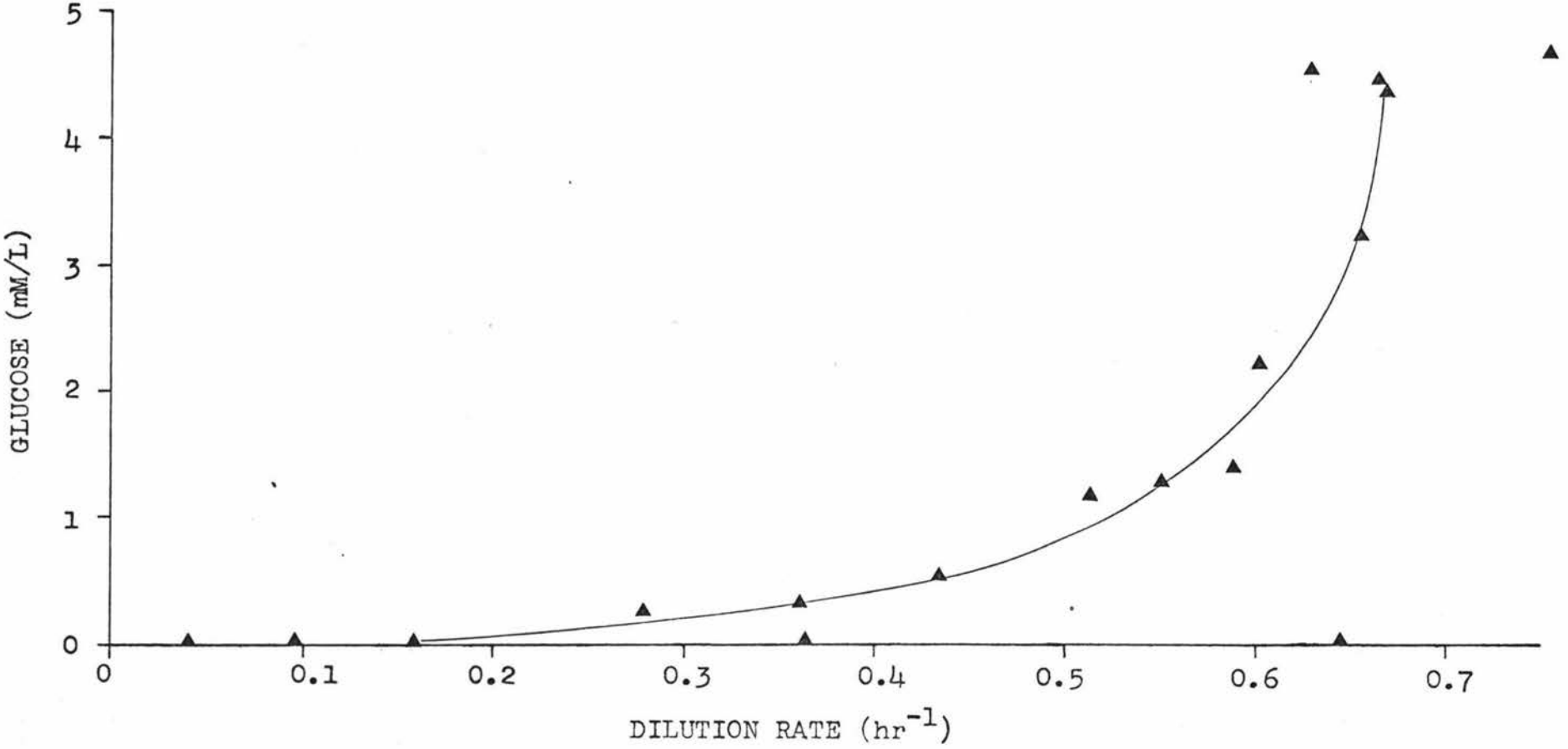

4
3
2
1
0
1/μ
0
1
2
3
4
1/S

Figure 18: Continuous Culture of WV1; Summary of Glucose Determinations


GLUCOSE (mM/L)
5
4
3
2
1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
DILUTION RATE (hr^{-1})

III.3. Mixed Continuous Culture of Sb1 & WV1

III.3.1. Establishment of inoculation procedure and results of mixed growth at moderate rate.

In preliminary experiments with mixed cultures it was thought that the optimal inocula ratio would be 1:1. In these experiments, WV1 was allowed to grow to stationary phase, the pump started, and a large batch culture of Sb1 (grown in a flask allowing anaerobic transfer, II.3.3.) was added until the desired ratio was attained. In the first of these, the transfer was poorly performed. The medium became oxidised (the indigo carmine turned blue) and the initial ratio of Sb1 to WV1 was 7:1, respectively, (by total cell count). WV1 was no longer visible microscopically after four volume exchanges. Specific growth rate was 0.355 hr^{-1}. This rate was chosen in reference to continuous culture results for WV1 complete consumption of glucose by WV1 and dense growth).

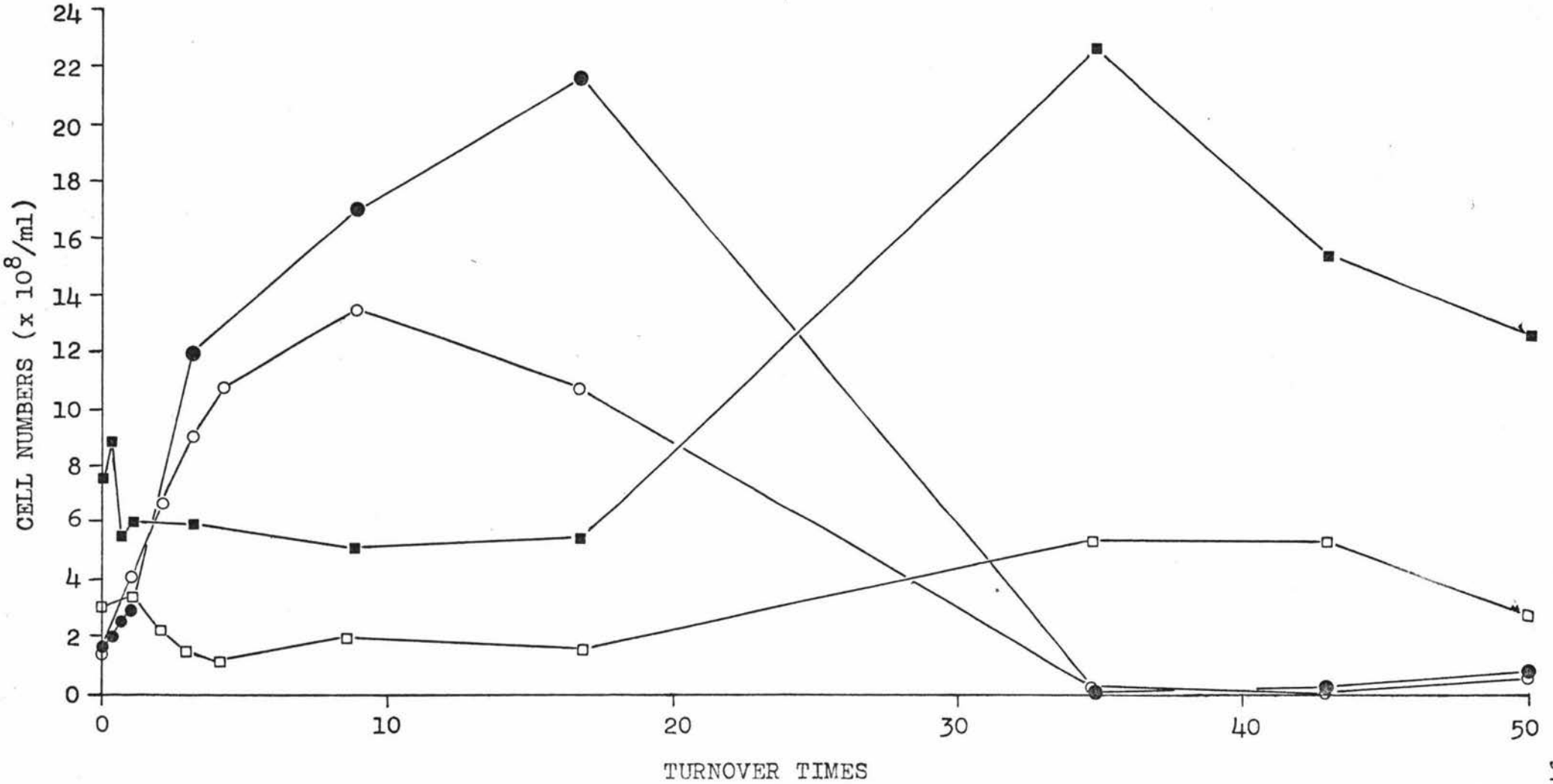
In the second experiment, the inoculation procedure was the same, but the medium in the growth vessel was partially oxidised (green color indicating approximately half reduction of indigo carmine, a potential of -129 milli-volts). The ratio of CFU at inoculation of Sb1 was 1:1. WV1 almost disappeared from the growth vessel after four volume exchanges (less than 10^4 CFU/ml, while Sb1 stabilised at 2.0×10^9/ml).

In the third experiment, inoculation was performed in the same manner, but the volume of culture transferred was reduced. The results of this experiment are shown in figure 19. Again the growth vessel was slightly oxidised, but not to such an extent as in the previous experiment. Sb1 dominated the culture within two volume exchanges, while WV1 was reduced, but did not disappear from the culture. Sb1 retained its numerical dominance for about twenty volume exchanges, after which the roles were reversed and WV1 showed numerical dominance. The last upward curve of Sb1 is not an

Figure 19: Mixed Continuous Culture of Sb1 and WV1 at D = 0.36 hr^{-1}

■ , total counts of WV1; □ , CFU of WV1;

● , total counts of Sb1; ○ , CFU of Sb1.


CELL NUMBERS (x 10^8/ml)
24
22
20
18
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
TURNOVER TIMES

indication that Sb1 would return to dominance because wall growth was noticeable at that time.

The results of a further experiment at approximately the same growth rate are shown in figure 20. The inoculum of Sb1 in this experiment, as in all subsequent experiments, was the contents of two standard 10 ml stock liquid cultures that had been incubated overnight. This procedure gave a consistent inoculum size and gave no noticeable oxidation of the medium in the growth vessel.

The curve in figure 20 shows an initial period of oscillation (0 to 20 volume exchanges) followed by a long period of fairly stable levels (30 to 70 volumes exchanges). During the latter period, WV1 dominates the culture by a factor of approximately two.

The results of a repeat experiment at a dilution rate of 0.36 hr^{-1} are shown in figure 21. The results for Sb1 are similar to those of the preceding experiment. The results for WV1 differ from the preceding experiment in initial cell concentration, the amplitude and duration of oscillation and the ratio of CFU to total counts. The last of these indicates an increased viability of WV1 over the course of the experiment.

III.3.3. Mixed Culture of Sb1 & WV1 at low growth rates.

The first experiment at a low growth rate was at a rate considerably below that intended. This was due to non-linear correlation of pumping speed to pump micrometer settings at low pumping speeds, due possibly to slight back flow through the one way valves. Because of this, the first mixed experiment had a low growth rate of 0.018 hr^{-1} (volume exchange time of 55.5 hours). After inoculation with Sb1, the culture was allowed to grow through five volume exchanges (eleven days). The results are shown in figure 22. During the first 1.5 volume exchanges, WV1 decreased in numbers. After 2 volume exchanges, both organisms maintained constant levels of growth. After 1

Figure 20: Mixed Continuous Culture of Sb1 and WV1 at D = 0.34 hr^{-1}

■ , total cell counts of WV1; □ , CFU of WV1;

● , total cell counts of Sb1; ○ , CFU of Sb1;

Z , represents the 95 % confidence limits.

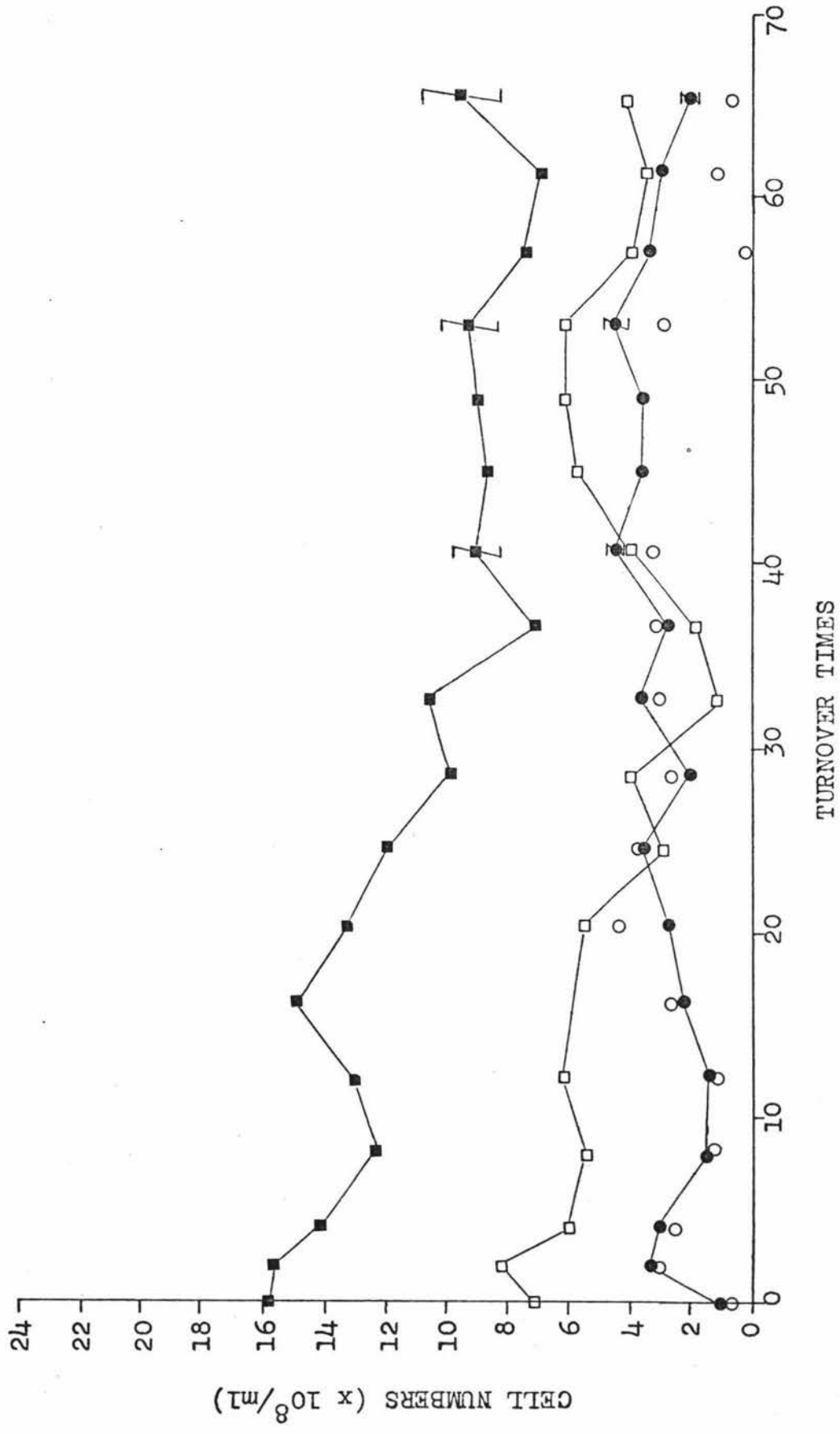

CELL NUMBERS (x 10^8/ml)
TURNOVER TIMES
0
2
4
6
8
10
12
14
16
18
20
22
24
0
10
20
30
40
50
60
70

Figure 21: Mixed Continuous Culture of Sb1 and WV1 at D = 0.36 hr^{-1}

■ , total cell counts of WV1; □ , CFU of WV1;

● , total cell counts of Sb1; ○ , CFU of Sb1;

⌇ , represents the 95 % confidence limits.

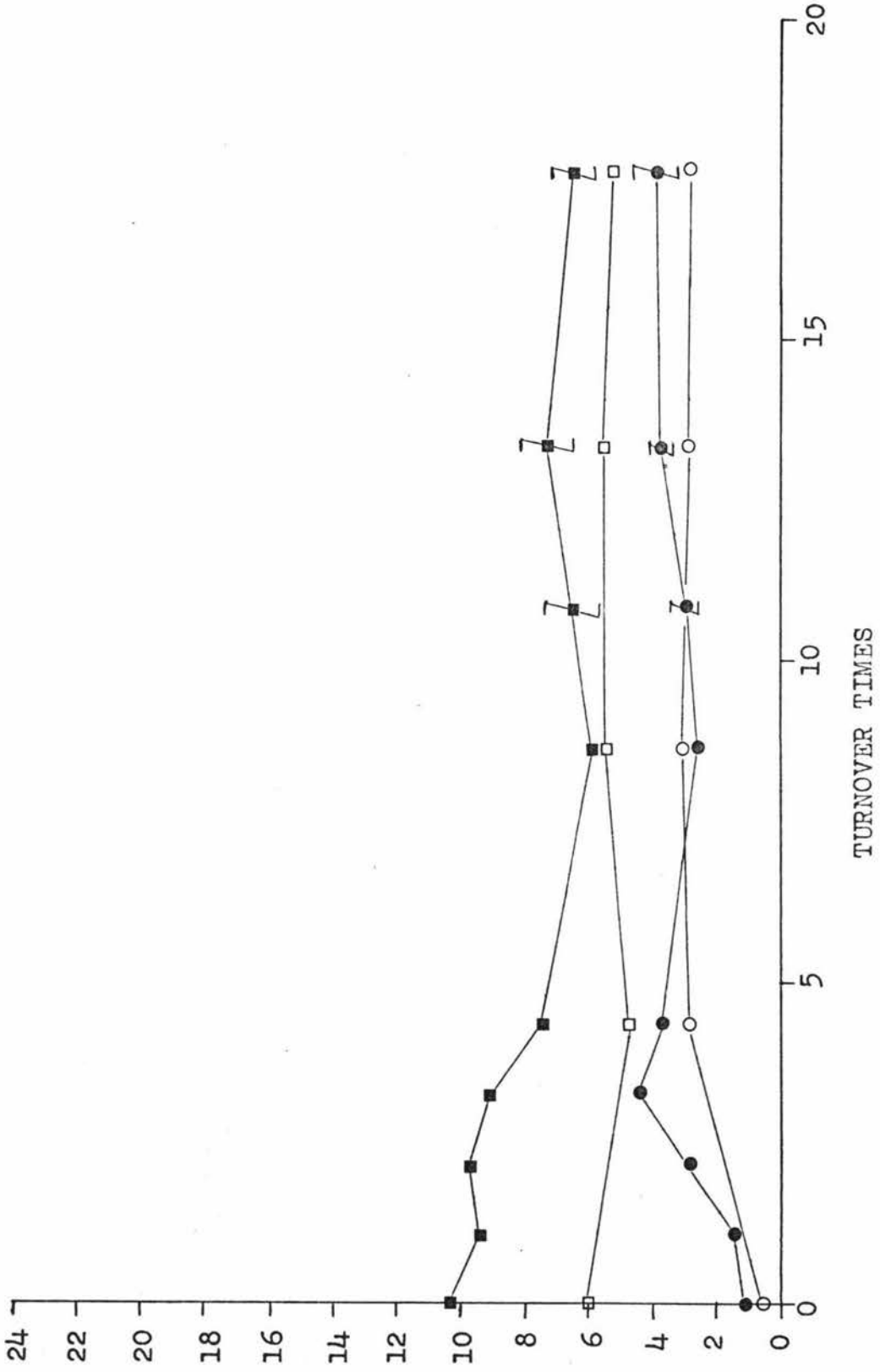
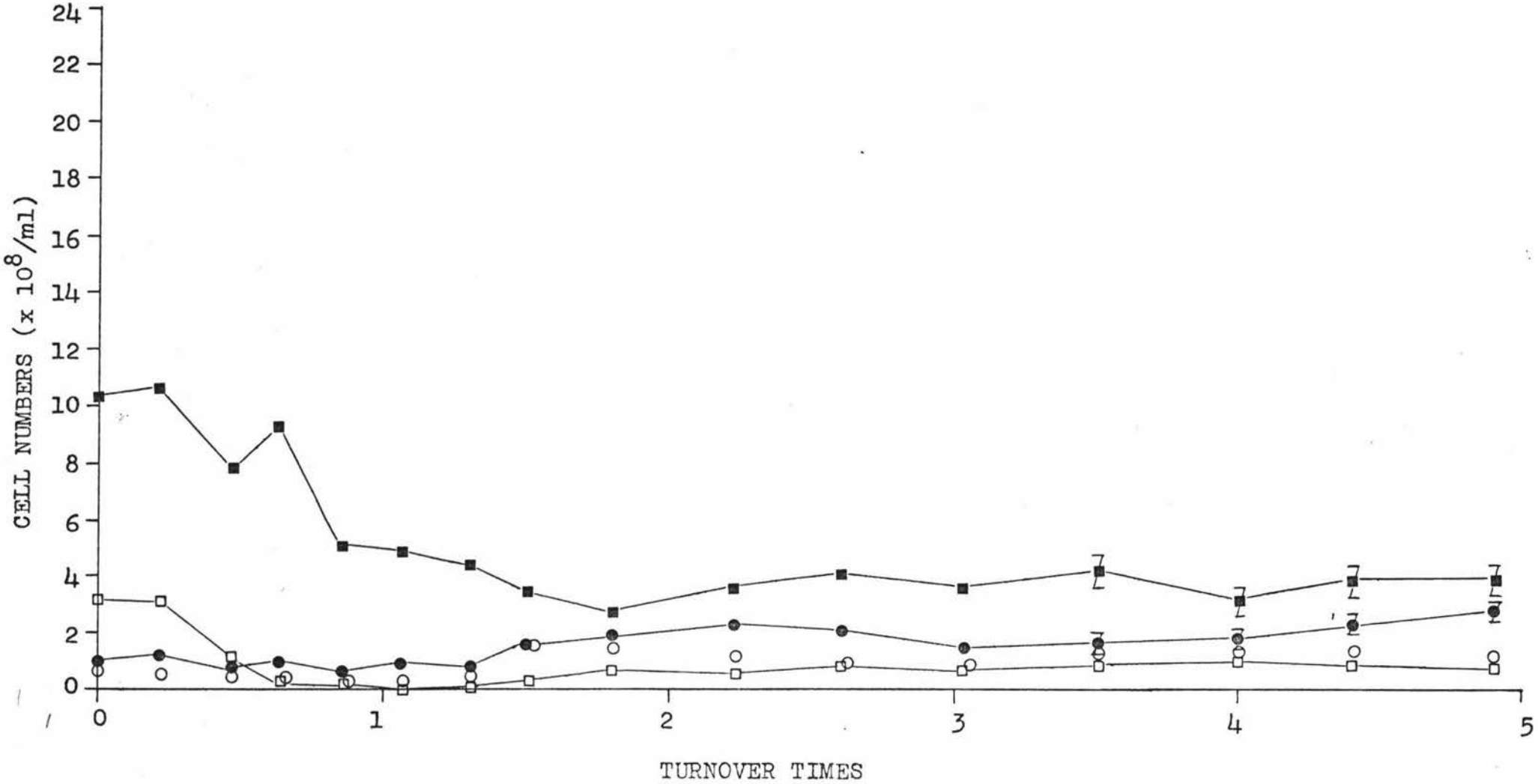


Figure 22: Mixed Continuous Culture of Sb1 and WV1 at D = 0.018 hr^{-1}

■ , total cell counts of WV1; □ , CFU of WV1;

● , total cell counts of Sb1; ○ , CFU of Sb1;

⌇ , represents the 95 % confidence limits.


CELL NUMBERS (x 10^8/ml)
24
22
20
18
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
TURNOVER TIMES

volume exchange, morphologically peculiar cells began to appear. (plate 11). The variant morphology of Sb1 was most notable, but a slight variation was also noted in WV1. Cells of WV1 had slight bulgings similar to, but not as great as, those shown in plate 8 (Co_2 limited growth.) The average viability of WV1 and Sb1 as measured by the ratio CFU/total counts is 0.227 and 0.547 respectively.

After five volume exchanges at 0.018 hr^{-1} , the dilution rate was increased to 0.052 hr^{-1} (volume exchange every 19.2. hours.) The results of this increase in dilution rate are shown in figure 23. The total count of WV1 increased during six volume exchanges, then decreased to a stable plateau at seven volume exchanges. Sb1 remained at approximately the same level throughout. The ratio of CfU to total cell count for WV1 decreased from 0.27 early in the experiment to less than 0,1 at the end of the experiment.

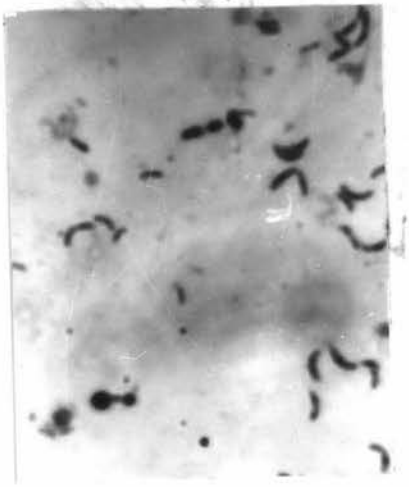
A red mass was noted hanging from the medium input at about the eleventh volume exchange. This was interpreted as a mass of Sb1, and the culture was allowed to continue for two days further to obsurve the effect of this on the culture balance. Sb1 continued to increase, apparently without reducing the numbers of WV1. The identity of the red mass with Sb1 was confirmed microscopically.

A repeat experiment was undertaken at a dilution rate of 0.058 hr^{-1}. In this experiment, the inoculum of Sb1 was two days old. The results of this experiment are shown in figure 24 (III.3.F.). At the fifteenth volume exchange, red growth was noted in the medium input tube, and the experiment was terminated.

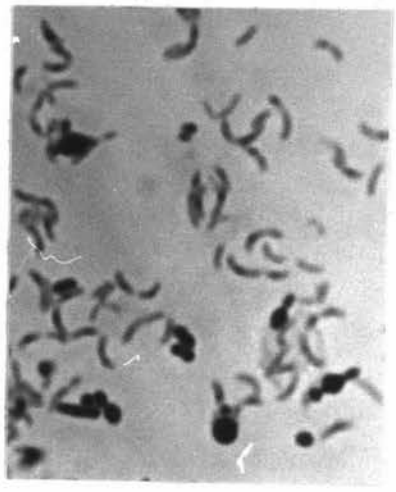
A final experiment at a dilution rate of 0.0575 hr^{-1} (volume exchange time of 17.4 hours), where the inoculum of Sb1 was four days old is shown in figure 25. In this experiment there is no initial increase of Sb1, presumably due to the age of the inoculum. The culture remained essentially static

Plate 11: Cells from Continuous Mixed Culture at a Very Low Growth Rate

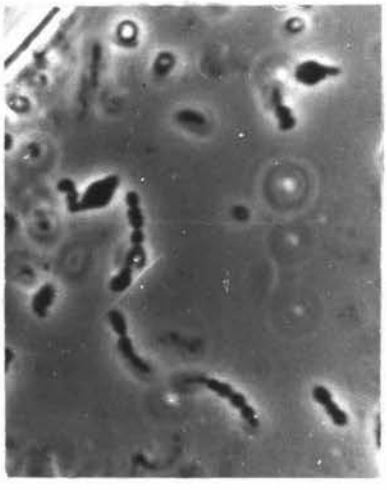
Plates 11A to 11F show photomicrographs of gram stains (except 11C, which is under phase microscopy) at differing times during growth at D = 0.018 hr^{-1} and after a rate increase (11E & 11F). The times during the experiment are as follows (volume exchanges): 11A, 0.85 (fig. 22); 11B, 1.5 (fig.22); 11C, 3.0 (fig.22); 11D, 4.0 (fig.22); 11E, 3.16 (fig.23); 11F, 6.95 (fig.23).



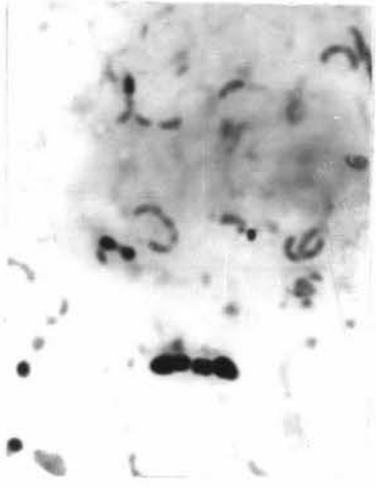
11 A



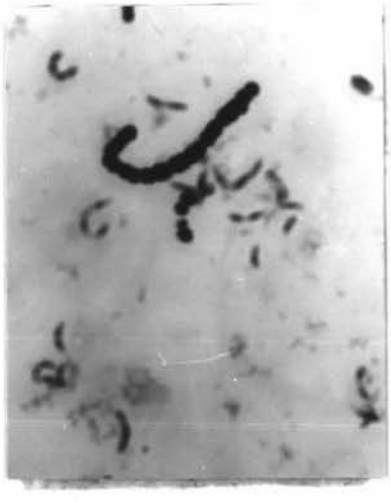
11 B



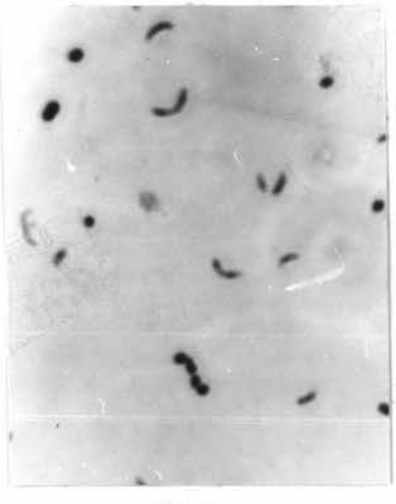
11 C



11 D



11 E



11 F

Figure 23: Mixed Continuous Culture of Sb. and SB1 at D = 0.052 hr^{-1}

■ , total cell counts of WV1; □ , CFU of WV1;

● , total cell counts of Sb1; ○ , CFU of Sb1;

⌇ , represents the 95 % confidence limits.

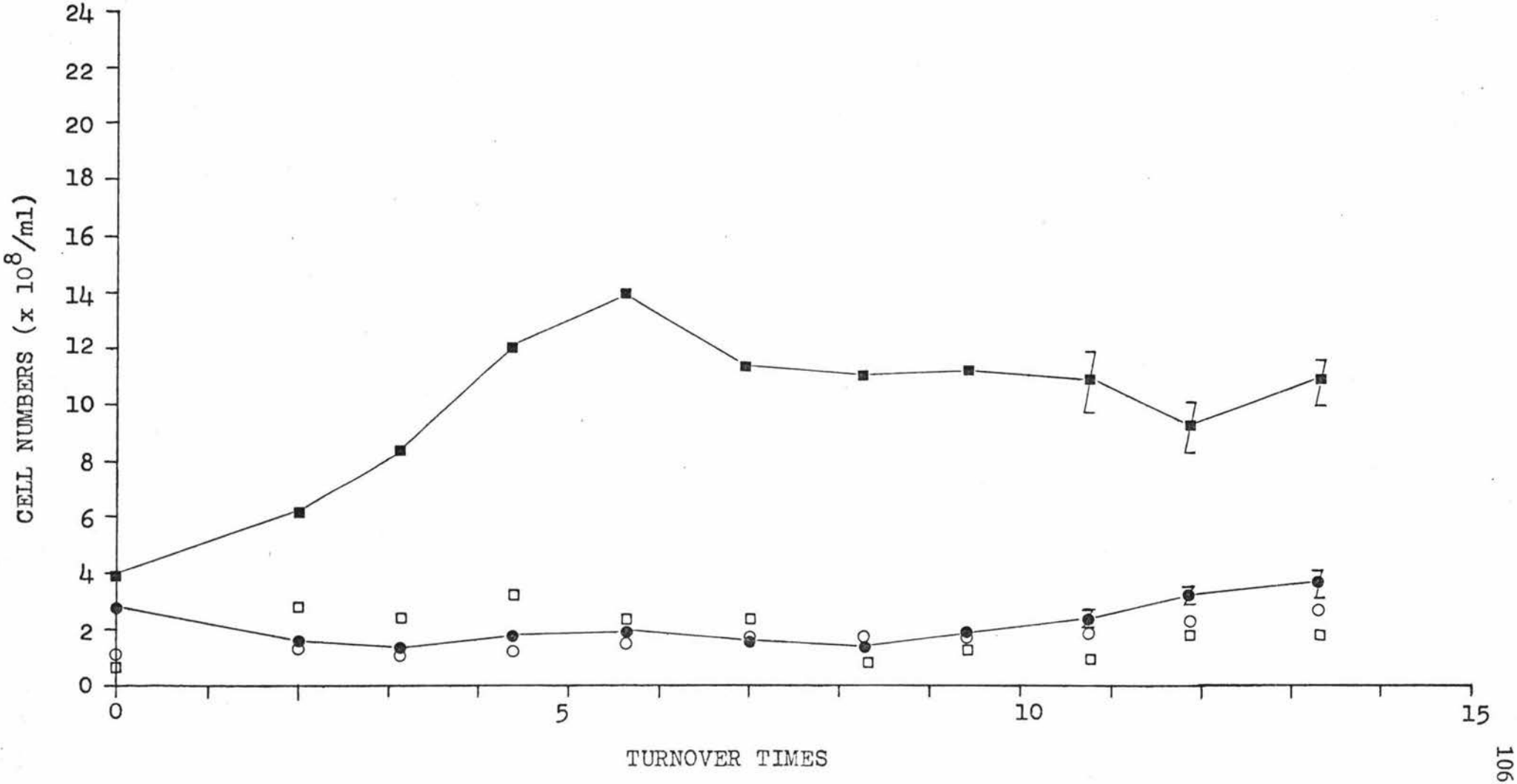

CELL NUMBERS (x 10^8/ml)
24
22
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
TURNOVER TIMES

Figure 24: Mixed Continuous Culture of Sb1 and WV1 at D = 0.058 hr^{-1}

■ , total cell count of WV1; □ , CFU of WV1;

● , total cell count of Sb1; ○ , CFU of SB1.

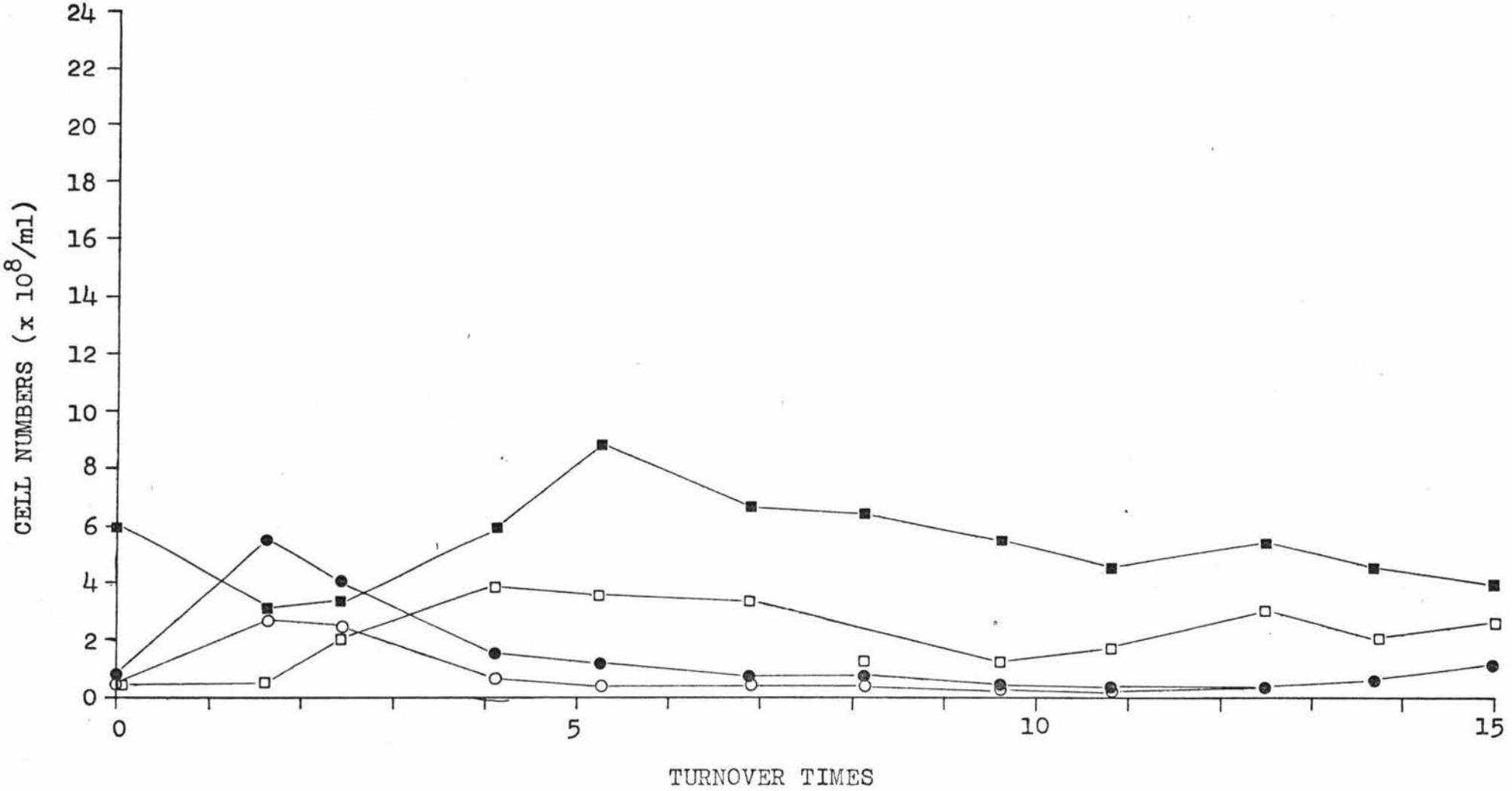

CELL NUMBERS (x 10^8/ml)
24
22
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
TURNOVER TIMES

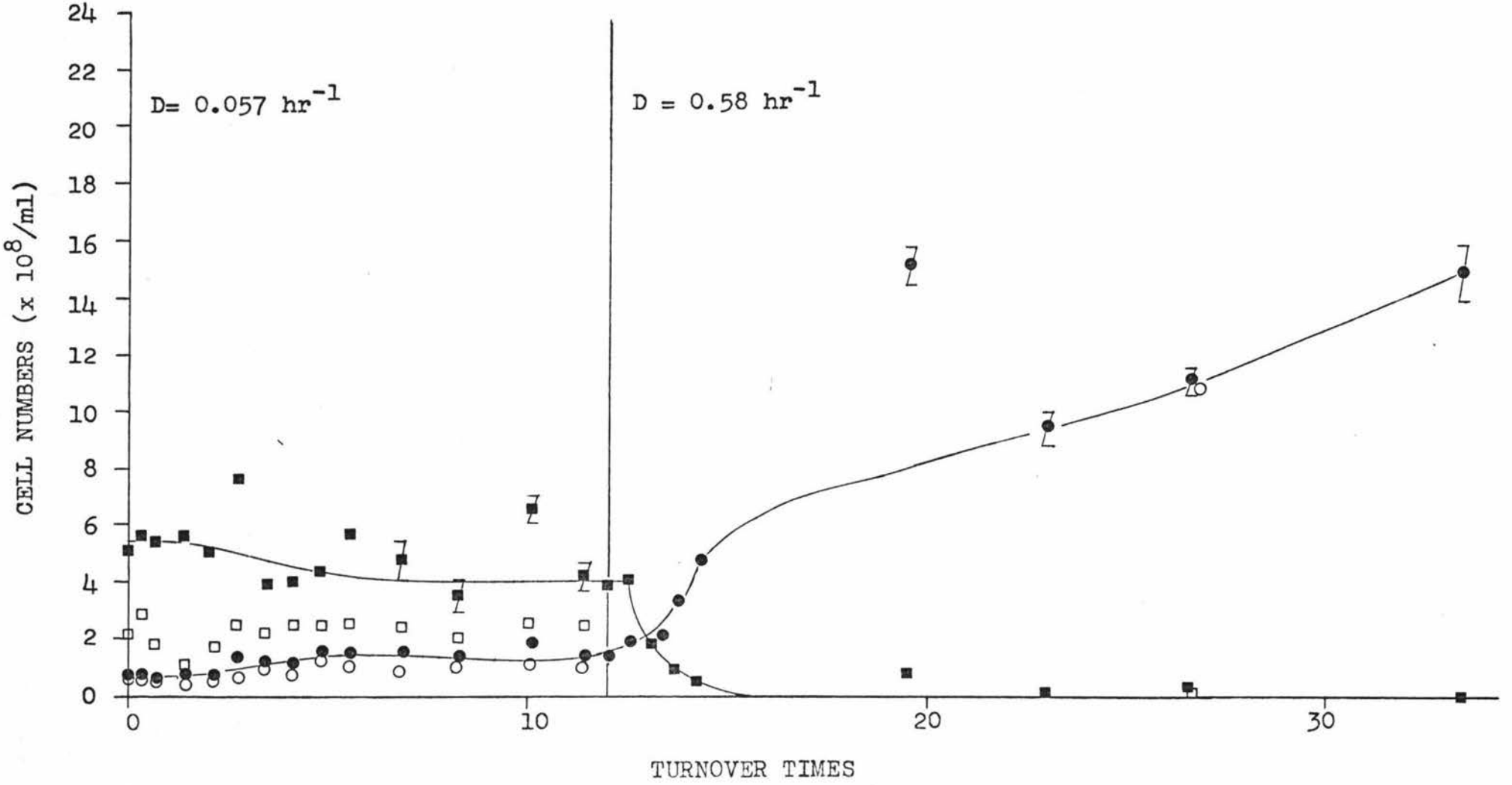
Figure 25: Mixed Continuous Culture of Sb1 and SB1 at D = 0.057 hr^{-1} with a shift up in growth rate.

The vertical line indicated the time at which the rate was shifted up.

■ , total WV1; □ , CFU of WV1;

● , total Sb1; ○ , CFU of Sb1;

Z , represents the 95 % confidence limits.


D= 0.057 hr^{-1}
D = 0.58 hr^{-1}
CELL NUMBERS (x 10^8/ml)
TURNOVER TIMES
0
2
4
6
8
10
12
14
16
18
20
22
24
0
10
20
30

during twelve volume exchanges. At that time, the rate of dilution was increased ten fold to 0.58 hr^{-1}. At this rate of dilution, Sbl rapidly increased as WV1 decreased in cell density.

IV. Discussion

IV.1. Culture Identities

The identity of Sb1 with Streptococcus bovis was confirmed by its high rate of growth, colonial and cellular morphology (Plates 1 &2), the production of primarily lactate from glucose (table 3), and by the tests of Fackham (1972).

The identity of WV1 with Butyrivibrio was confirmed by its colonial and cellular morphology (Plates 5,6,7 & 9) and the production of large amounts of butyric and formic acid from glucose (table 7).

IV.2. Batch Cultures

Molar growth yields for Sb1 and WV1 grown with glucose as determined from batch cultures are 40.2 g / M and 55 g / M, respectively (figs. 5 & 9). The cell yield of Sb1 is approximately twice that expected from a homofermentative organism and would indicate, using the constant of Bauchop and Elsden (1960), a yield of nearly four ATP per glucose by Sb1. Assuming the same constant, WV1 achieves greater than five ATP per glucose. That Sb1 is still behaving as a homofermenter is shown by the levels of lactate produced, usually 85 to 90 % of the maximum possible from the substrate.

High yields such as these have been observed before and have been explained by retentions of substrate as reserve material (Hungate, 1963), by postulating additional ATP production (Hobson & Summers, 1972) or by postulating a greater synthesis of cell mass per ATP than did Bauchop and Elsden (Haukeli & Lie, 1971). Efforts to discover reserve material failed with both organisms, and a capsule was only present on Sb1 (Plate 2). The capsule could possibly absorb some un-metabolised glucose, but this was not observed in the autoradiograms of acid hydrolysates of ^{14}C glucose fed cells. Acid hydrolysis is fairly destructive of sugars (Basham & Calvin, 1957) so that this is not conclusive.

To determine whether some other element in the medium was contributing to cell yield, growth with varying levels of trypticase plus yeast ex-

tract was measured (figs. 8 & 10). The level of trypticase strongly affected the cell yields of Sb1 and WV1. The Ks for each was 0.046 g / l and 0.38 g / l, respectively, which implies that Sb1 is eight times more efficient at absorption of growth factors from trypticase or yeast extract. For both organisms, the increase in yield with increasing trypticase levels was nearly linear for concentrations of trypticase greater than 2 g / l. An attempt was made to estimate a yield at zero trypticase by extrapolating along this line to the y-axis and estimating yield from the dry weight at the intercept. This gave estimations of yield for Sb1 and WV1 of 45 g / M and 68 g / M, respectively, at zero trypticase. Both these figures are above the estimations made from growth in glucose limited medium. These high yields may have geen due to the presence of low concentrations of acetic acid in the trypticase. Steam distillation of uninoculated medium, and thin layer chromatography of the steam distillate, showed that a small amount of acetic acid was present in the trypticase. This could be one factor contributing to the increased yields. That the estimates of yield in these experiments were higher than the previously determined average could be due to the manner of addition of trypticase to the medium. In these experiments, it was added dry to the flasks and the remaining constituents of the medium having been boiled and equilibrated to 39 C. under CO_2, were added and the flask sealed. This meant that the trypticase was not boiled as it was in standard medium and would have retained more acetic acid.

Carbon dioxide is known to affect strongly the growth of rumen bacteria and other anaerobes (Caldwell et al, 1968; Watt, 1973; Dehority, 1971). There have been few measures of the molar growth yield with CO_2 (Buchanan & Pine, 1963). Sb1 had no absolute requirement for CO_2, but in the absence of CO_2, the molar growth yield was 16.5 (table 3). This is less than the yield of 21 expected from a homofermentative organism on the basis of the Bauchop & Elsden constant. This discrepancy is anomalous, becauselactate production appeared to account for nearly

100 % of the glucose present. It is noteworthy that yield of Sb1 is maximal and yield of WV1 is very nearly maximal when CO_2 is equimolar with glucose. Whether this is a significant ratio or not would require measurement of cell yield with CO_2 at other concentrations of glucose. The probable significance of this ratio is supported by the $^{14}CO_2$ and ^{14}C-glucose labelling experiments (tables 6 & 12). Approximately the same percentage of label from each compound is retained by both organisms, i.e., about 10 % when CO_2 and glucose are initially equimolar.

The experiment measuring decrease of ^{14}C-glucose label and dry weight of cells after stationary phase (III.1.1.5. & III.1.2.5.) showed an 18 % decrease in label and a 13 % decrease in dry weight for Sb1 and a 30 % decrease in label and an 11 % decrease in dry weight for WV1 24 hrs after the beginning of lag phase. This is a decrease in activity per dry weight of 6 % for Sb1 and 21 % for WV1. The reduction in Sb1 is too little to be of wignificance while that in WV1 could mean that some of the label was in the form of an energy reserve and was lost to the medium during stationary phase.

The distribution of label between fermentation products of Sb1 (table 8) indicates that a significant amount of glucose is converted to acetate and a small amount appears in formate. The actual amounts produced cannot be determined from these results because of low recoveries, so that estimates of the amount of substrate used for production of acetate and formate cannot be made. The fact remains that they are produced, and probably energy for synthesis of cell material is derived from their production. A small portion (0.5 % of the total label) of the $^{14}CO_2$ labelled culture of Sb1 appears as formate and this is considered to be insignificant.

Results from thin layer chromatography of supernatant from ^{14}C labelled CO_2 and ^{14}C glucose fed cells of WV1 (table 14) indicate an equimolar production of formate and butyrate from glucose. The most significantly labelled volatile fatty acid from $^{14}CO_2$ labelled

cultures is formic acid. This indicates either a role for CO_2 as a proton acceptor or that CO_2 and formate are exchangeable at some point in their metabolism. To distinguish between these possibilities, timed experiments on the appearance of label in formate in an actively growing culture would be necessary. That CO_2 is a proton acceptor is more likely because, if it were exchanging with free CO_2, the level of ^{14}C formate from glucose labelled cultures would be less. However, energy generation as a result of formation of formate from CO_2 has not been previously reported.

The nature of the incorporated glucose label in Sb1 and WV1 is shown from the results of autoradiograms of thin layer chromatograms of acid hydrolysates (tables 9 & 15, respectively). Label in Sb1 only appears in amino acids. Roughly one half the glucose label in WV1 appears in amino acids, a small amount in a nucleic acid, and half in material which chromatograms like a neutral compound, the nature of which was not determined.

The significance of these results in relation to the estimation of yield from these organisms is that neither Sb1 or WV1 behaves ideally in respect to the Bauchop and Elsden constant. Both organisms retain about 10 % of the substrate. Sb1 is primarily, but not completely homofermentative under the conditions of growth used in this work. WV1 may retain a portion of the substrate as a reserve material represented by the neutral compounds in the autoradiograms CO_2greatly affects the yield of Sb1 while not greatly altering the products of fermentation. If the amount of label retained by Sb1 is taken as 9 %, the maximum increase in weight of Sb1 due to the incorporated CO_2 in the form CH_2O is only 15 mg / 1 dry weight of cells. This raises the Yg in the absence of CO_2 from 16.5 to 19.2 g / M, which is far short of the observed yield of 40 g / M in the presence of CO_2.

Growth of WV1 with CO_2 in the presence of 5.55 mM glucose shows an almost linear increment as the concentration of CO_2 increases. For the

concentrations of CO_2 of 1.11 mM / 1, 2.78 mM / 1 and 5.55 mM / 1, the ratios of dry weight to mM of CO_2 are 41.2, 67.0 and 45.8, respectively (average equals about 51). For a single experiment, these values are sufficiently close to the average molar growth yield from glucose (55 g / M), which was determined in excess CO_2, to be taken as equivalent.

IV.3. Continuous Monocultures of Sb1 and WV1

The Monod growth constants, Ks and μ_{max}, of Sb1 and WV1 as determined from continuous cultures of those organisms were 0.429 mM / 1 and 2.47 hr^{-1}, and 0.332 mM / 1 and 0.704 hr^{-1}, respectively. The Ks constant of WV1 is slightly less than that of Sb1, implying a greater substrate affinity for glucose by WV1 (Veldkamp, & Jannasch, 1972). These values are below the level of substrate that could have heen used to determine these constants by use of batch culture, as no visible growth would have occurred by the methods available. The μ_{max} determinations are in agreement with the maximum rates observed in batch culture.

If the curves summarizing dry weight determinations (figs. 13 & 16) are contrasted, it can be seen that WV1 is present in greater mass up to growth rates of 0.55 hr^{-1}, above which Sb1 is present in greater mass. The Yg estimates from all equilibrium values for Sb1 and WV1 were 39.6 $\pm$ 7.6 g / M and 69.1 $\pm$ 6.8 g / M, respectively. The estimate of Yg for Sb1 is very close to that determined from batch cultures (40.2 g / M) while that of WV1 is considerably greater than the 55 g / M determined from batch cultures. The latter result has been encountered before (Hobson, 1964) where the quantitative effect of CO_2 was not considered and the yield of S. bovis was not calculated. Hobson and Summers (1972) suggested that it is preferable to explain the high Yg observed in continuous cultures of Selenomonal ruminantium by electron transfer reactions which generate additional ATP subsequent to pyruvate in the catabolism of glucose.

If the rumen is considered as a continuous culture, the dilution

rates of interest are those less than 0.1 hr^{-1}, as the estimated turnover time of the rumen is about 18 hr (Hungate, 1966). For an 18 hr turnover time, D = 0.055 hr^{-1}. Very few determinations of growth parameters were made in this region for Sb1, which is unfortunate, since the numbers of Sb1 under equilibrium conditions at growth rates near D = 0.055 hr^{-1} are reduced. This is the region where maintenance energy requirements would appear to he having a significant effect on yield (Stouthamer & Bettenhaussen, 1973), but the number of determinations is too few to apply the mathematics for estimation of maintenance energy rates (Marr et al, 1963). Dry weight estimations in this region did not significantly decrease below those observed at D greater than 0.2 hr^{-1} for either organism. Estimations of colony forming units and total cell counts decreased below D = 0.2 hr^{-1} with Sb1 while no significant decrease appeared for WV1 down to D = 0.04 hr^{-1}.

It has been shown that organisms from a low glucose environment that are placed in a medium rich in glucose give lower counts than if the medium is low in glucose, and this has been termed substrate accelerated death (Postgate, 1973). The possibility that CFU would be decreased by substrate accelerated death is eliminated by the fact that the total counts are also reduced, and the ratio of total count to CFU is constant. A larger number of determinations of dry weight in this region might have given a more significant curve for the relationship of dry weight to dilution rate.

Contrasting Sb1 and WV1 in terms of CFU and total counts shows that Sb1 produces about 7.0 times as many CFU as WV1 but only 1.2 times as many total counts. The possibility of substrate accelerated death of WV1 is eliminated by the constancy of the ratio of total counts to CFU counts for WV1 at higher growth rates, where glucose is still present in the growth vessel.

It thus appears that viability is constant for both bacteria for all dilution rates, and variations in the mathematics of continuous culture

due to lowered viability (Sinclair & Topiwala, 1970; Tempest, 1970) can be ignored.

The possible role of the red pigment produced by Sb1, which has been noted as constitutive in some strains of S. bovis (Ota &Kitahara, 1971), in CO_2 metabolism was noted from its association with rapid growth rates in continuous culture and in frequently subcultured batch cultures, and its absence in CO_2-free cultures, but this is far from conclusive.

IV.4. Continuous Mixed Cultures

Results from mixed continuous cultures show that the best inoculum for the early establishment of equilibrium is an old culture of Sb1. If a fresh culture of Sb1 is the inoculum, large oscillations may occur, which require many more turnover times to equilibrate than do monocultures.

In mixed culture, the total counts and CFU of WV1 at D = 0.36 hr^{-1} are very nearly the same as in continuous mono-culture, while at D = 0.055 hr^{-1}, CFU are reduced by about one half and total counts by about two thirds those observed in monoculture. Total counts and CFU of Sb1 in mixed culture are reduced to one eiththh of those in continuous monoculture at D = 0.36 hr^{-1}, as are total counts of Sb1 at D = 0.055 hr^{-1}. CFU of Sb1 at D = 0.055 hr^{-1} in mixed culture are reduced to one quarter the number appearing in continuous monoculture. At both rates, after equilibrium is established, WV1 is more numerous than Sb1 by estimation of CFU and total counts by a factor of at least 2.5. WV1 in mixed culture also deviates less from its levels in monoculture than does Sb1.

The explanation of dominance of WV1 over Sb1 at rates near 0.36 hr^{-1} could be explained solely by the greater yield of cell mass per unit substrate. This does not explain why Sb1 is not completely excluded from the culture at this rate. At the lower growth rate of 0.055 hr^{-1}, numerical superiority of WV1 could be due to both greater yield per unit of substrate and a lower maintenance energy requirement. In

Bacillus species, cell wall turnover is necessary for normal growth and the energy required for this process gives Bacillus species a high maintenance energy requirement (Stouthamer & Bettenhaussen, 1973). That Sb1 has a higher maintenance energy requirement than WV1 is indicated by the drop in numbers of Sb1 below $D = 0.2\ hr^{-1}$ while those of WV1 do not decrease below this rate. That the higher maintenance energy may be due, at least in part, to a higher rate of cell wall turnover is shown by the morphologically peculiar cells of Sb1 found at $D = 0.018\ hr^{-1}$ in mixed culture (Plate 11).

Continuous mixed culture of rumen bacteria has shown that cross feeding can occur between bacteria (Hover & Lipari, 1971) and that culture mixing can cause changes in the observed end products of fermentation (Iannotti et al, 1973; Scheifinger & Wolin, 1973). Since the growth of Sb1 is depressed in mixed cultures, the growth relationship does not correspond to the former. The possibility that mixed growth alters the fermentation products of one or both organisms was not tested.

IV.5. Conclusions

The original hypothesis to be tested in this work was whether dominance of one bacterium over another could be explained on the basis of relative efficiency of energy yielding metabolism, since, if both organisms used the energy with about the same efficiency to synthesise new cell material, the organism which generated the most energy per mole of substrate would produce the most cell material. If this hypothesis were true, and Sb1, as a homofermenter, derived two ATP per mole of glucose and WV1 derived a larger number, then WV1 would dominate the continuous mixed cultures, unless the rate of growth were increased to a level where WV1 did not consume all of the substrate (fig. 25). At this rate of growth, the superior maximum growth rate of Sb1 allows it to out-compete WV1.

The possible role of Ks cannot accurately be assessed because the

estimation of S in continuous cultures of Sb1 showed too great a scatter and there was a positive curvature in plots of measures of growth with D at high growth rates, and hence the value of the Line-weaver-Burke plot (fig. 14) for estimating Ks was reduced. A more direct measure of substrate affinity would be necessary to determine the relation of this with dominance in mixed culture. If the Ks of WV1 is lower than that lf Sb1, then WV1 falls in the class of organisms observed by Jannasch (1967) which out-compete high Ks organisms when S is low due to higher substrate affinity.

The growth yields of Sb1 and WV1 were greater than expected, but similar to yields observed previously. The reason for enlarged yield was found to be due to the stimulatory effect of CO_2 and hence the probable derivation of more ATP per mole of glucose in the presence of CO_2, which has been previously observed in Actinomyces israeli (Pine &Howell, 1956; Buchanan & Pine, 1963) and is consistent with the views of Payne (1970) that the Bauchop & Elsden constant is a true constant and that novel means for the generation of ATP should be sought.

There remain three possible explanations for the dominance of WV1 over Sb1. WV1 dominates because it derives a greater cell yield per unit of substrate, because of a lower rate of maintenance metabolism, or because it has a greater affinity for the substrate (a lower Ks). More measures of growth parameters in monoculture and mixed culture at low growth rates would be necessary to judge the significance of the second possibility, while greater accuracy of measure of continuous culture parameters of Sb1 at high growth rates would be necessary for the last propasition. None of these explain the formation of a stable equilibrium as observed in continuous mixed cultures.

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