STUDIES ON BACTERIAL PREDOMINANCE PATTERNS IN MIXED CULTURES

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CHAPTER I

INTRODUCTION

A. General

In modern times, the enormous volumes of sewage and industrial wastes produced by our vital and industrial processes are treated and purified by biological treatment systems. In essence, all waste water treatment processes now being utilized consist of placing the sewage or industrial waste in contact with a microbiological population which is capable of metabolizing the organic matter contained in the waste waters. In the trickling filter process, the waste water is spread over layers of crushed rock which are covered with a dense biological growth. In activated sludge, the waste water is placed in contact with a heavy biological growth or sludge, and agitated in the presence of air. In both processes, after the period of contact, the liquor is settled to remove the biological growths, and the purified supernatant is decanted. Because of its efficiency, as well as its flexibility, the activated sludge system and its various modifications are considered to be the process of choice in modern waste treatment.

In the treatment processes now operational, the biological populations which actually perform the purification of the waste are of an undetermined nature. In activated sludge, it is well known that a heterogeneous microbial population constitutes the sludge, but the population itself is, in most cases, undefined and uncontrolled. In such continuous flow systems, where many species of microorganisms exist in a state of continuous competition with each other, an excellent opportunity arises to study the dynamics of population changes.

B. Scope and Objectives

The research reported in this thesis involved the selection and study of several bacterial species isolated from sewage or known to be present in sewage. These bacterial species were studied individually to determine their growth characteristics and biochemical behavior. Subsequently, experiments were performed on mixed cultures in continuous and discontinuous systems, and their behavior was examined in detail.

Although activated sludge contains a varied microbiological population, the studies reported in this thesis were limited to interaction between bacterial species. No attempt was made in this work to extend the study to more extensive fields of research, such as bacteria-protozoa, or bacteria-bacteriophage interaction. It is to be noted, however, that all such relationships do play a part in predominance patterns of activated sludge.

The primary objective of this research was to attempt to gain an insight into the mechanism of bacterial interactions in mixed systems. A basic research approach to the problem was adopted, because of the lack of knowledge in the area of mixed culture interactions provided by the basic sciences of microbiology and biochemistry. A secondary objective of this work was to relate the mechanisms studied and the knowledge gained to the practical field of bioenvironmental engineering.

C. Engineering Justification

Bioenvironmental engineers, a new breed of professional men combining the knowledge of the basic biological and physical sciences with the practical resources of engineering, are now called upon to bear the brunt of the battle for the conservation of our water resources and the reduction of the pollution problem facing the nation. These men are called upon to design new and more efficient treatment facilities, to operate them in a more efficient manner, and to perform research that will give us new and improved treatment processes. One field in which fruitful research would be of great importance in providing for better waste treatment is that of determining factors responsible for species predominance.

In activated sludge reactors, the heterogeneous microbial population is known not to have a steady composition, but to change from time to time in a seemingly random manner. In certain cases, massive changes in predominance

are observed, and these may have deleterious effects on the quality of the treatment. An example of this type of predominance change is that producing filamentous organisms resulting in bulking sludge. Very few attempts are usually made to select the population in the reactors, to improve it, or to prevent changes harmful to the quality of the treatment.

If research into the field of microbiological associations were to result in an understanding of the mechanisms involved, the direct result could be an improvement of the biological treatment processes. Such research is definitely needed, and since for the most part it has not been and is not now being performed in the basic sciences, it is necessary that these investigations be conducted by the scientists directly associated with waste treatment. If engineers were able to select specific microbiological populations in treatment reactors to maximize the efficiency of the treatment by maintaining the optimum desirable population, the entire waste treatment field might undergo a veritable revolution leading to a more efficient production of the principal product that we are striving to obtain -- clean water. It is with this aim that the work herein reported attempts to make inroads into the understanding and control of species predominance.

CHAPTER II

LITERATURE REVIEW

A. Review of the Literature in the General Area of Species Predominance

Pasteur, shortly after discovering the first known obligate anaerobe, Vibrion butyrique, was the first scientist to recognize the principle of microbial association as applied to the coexistence of aerobic and anaerobic microorganisms (1). In 1863, he pointed out that, in nature, Vibrion butyrique and similar organisms must often depend upon the abstraction of oxygen from their environment by aerobic microorganisms in order to grow. Pasteur's explanation of the relationship of aerobic-anaerobic organisms was accepted by Roux (2), who utilized hay bacillus as a technical aid in the cultivation of Vibrion septique.

DeBary (1) in 1879, was probably the first investigator to emphasize the significance of the antagonistic relationships of microorganisms by observing that when two organisms were grown together on the same substrate, one was found to overcome the other. This relationship was designated by Ward (1) in 1899 as "antibiosis."

Following the pioneer work of Pasteur, Roux and Debary, numerous investigators have studied the effect of mixed populations in relation to various fermentation processes. Sherman and Shaw (3) found that the rate of fermentation of lactose to propionic acid was speeded up greatly by the combination of Streptococcus lacticus or Lactobacillus casei with Bacterium acidipropionici. Sturges and Pettger (4) noted that pure cultures of Clostridium putrificum grown in egg-meat medium showed greatly delayed putrefaction, frequently twenty to thirty days elapsing after seeding, under anaerobic conditions. However, these investigators noted that, when growing with another organism, the putrefactive change was manifested very promptly. No identification was made of the type of organisms that would induce rapid putrefaction in conjunction with Clostridium putrificum, and the authors indicated only that it could be an aerobe.

Additional work on microbial relationships was reported by Hutchinson and Clayton (5), who indicated that the products of cellulose decomposition stimulate nitrogen assimilation by Azotobacter. Sanborn (6) did additional work dealing with the factors which influence the rate of cellulose fermentation, and found that a cellulose-destroying fungus, Cellulomonas folia, was stimulated in growth by association with several other microorganisms. These organisms were identified as Bacillus subtilis, Bacillus mycoides, Bacillus cereus, and organisms of the genus Azotobacter. Sanborn explained these symbiotic

relationships by indicating that Bacillus subtilis, Bacillus mycoides, and Bacillus cereus secrete substances which function as essential food factors for Cellulomonas folia. These essential food substances were not identified. Also, Sanborn indicated that the growth stimulation with Azotobacter was brought about by the living cell, these organisms being able to synthesize an accessory growth factor similar to vitamin B. No identification of the co-factor was made.

Waksman (1) described the extent of development of any one group of microorganisms inhabiting natural substrates such as soil, compost, or sewage, as depending upon a number of factors as follows:

1. Food supply, both qualitative and quantitative. The presence or absence of certain specific nutrients favorable to the development of particular organisms would markedly affect their growth.

2. Environmental conditions, favorable or unfavorable to the development of the specific organisms, notably temperature, oxygen supply, moisture content, soil structure, and abundance of organic matter.

3. Presence and abundance of other organisms which may produce substances that are either stimulating or toxic to the organisms in question or which may compete with them for the available nutrients.

4. The presence of other organisms which are parasitic or phagocytic upon the particular forms. The role of

protozoa in controlling bacterial activities by consuming the cells was given as an example of these activities.

In order to explain the phenomenon of antibiosis in microorganisms, Broom (7) proposed that the exhaustion of essential food materials by the stronger strain was its cause. Waksman and Foster (8) studied different soil organisms and found among them fungi, actinomyces, and bacteria that are capable of producing, when grown on synthetic media, substances which are antagonistic to the growth of other soil organisms. A study was made of the antagonistic effect of one species of actinomyces upon a variety of fungi, bacteria, and other actinomyces, and it was found that the inhibiting effect produced by this organism was not due to exhaustion of nutrients or to unfavorable changes in reaction, but was a specific effect of one strain on the other.

Holman and Meekison (9) indicated that a considerable number of pairs of organisms could produce gas from carbohydrates or polyatomic alcohols; however, neither one of the pair was able to bring about the change alone. They applied the term "synergism" to this type of association in which two bacteria, growing together, brought about effects which either of them growing alone was unable to accomplish. Lederberg (10) defined syntrophism as the growth of two distinct biochemical mutants in mixed culture as a result of the ability of each strain to synthesize the growth factor required by the other.

Rahn (11) noted that pure cultures in nature were very rare, and that, generally, nature works with mixed cultures, and that all natural fermentations, decompositions, and putrefactions were accomplished by a number of different species among which perhaps one dominated, but was influenced by the rest. He mentioned that the results obtained with pure cultures were not sufficient to explain all microbial activity in nature. Rahn also distinguished the three main cases of mutual influence between different organisms: symbiosis, where two organisms profit by the combination; metabiosis, where one profits by the other without benefiting the other in return; and antibiosis, where one organism injures the other. Fawcett (12) in an address to the twenty-second meeting of the American Phytopathological Society pointed out that classical research procedures with pure cultures were not completely satisfactory, because nature works most frequently with microbial associations. He indicated the necessity of entering actively into the investigation of the effects of known mixtures in comparison with the effects of individual organisms alone in their relation to plant diseases.

Savastano and Fawcett (13) using citrus fruits, determined that inoculation with certain mixtures of spores greatly increased the rate of decay, while when inocula containing certain other spore mixtures were used, the rate of decay was lower than when any one of the components was used alone. In other cases, mixtures were used for which

the decay rate approximated that of the most rapid component acting alone. Waksman and Hutchings (14) made a study of the associative growth of different fungi, actinomyces, and bacteria upon plant material. It was found that the presence of one organism could modify considerably the growth of another. The decomposition of alfalfa by a Trichoderma was modified by the presence of various fungi and bacteria that, although unable to decompose cellulose, were capable of favoring the decomposition of the cellulose by Trichoderma. It was also found that corn stalks could not be attacked by pure cultures of actinomyces. However, when certain fungi were previously grown upon the stalks, the growth of actinomyces was greatly favored. In addition, it was found that lignin decomposition took place only when actinomyces were present in a mixture of organisms.

Speakman and Phillips (15) found that the contamination of cultures of Clostridium pectinovorum with Bacillus volutam transformed the acetone-butyl alcohol fermentation of Clostridium pectinovorum to a lactic acid fermentation. Waksman and Lomanitz (16) found that Bacillus cereus was unable to attack alanine and phenylalanine, while glutaric acid and asparagine were acted upon to a limited extent, and casein and other native proteins were decomposed very rapidly. Bacterium fluorescens was unable to decompose casein but was able to utilize the amino acids contained in the protein. By combining the two organisms in casein media, the protein was decomposed very rapidly to ammonia,

Bacillus cereus hydrolyzing the casein to amino acids, and Bacillus fluorescens decomposing the latter to ammonia.

Buchanan and Fulmer (17) have reported on a very interesting symbiotic relationship where Azotobacter has been found growing associated with algae. They suggested that Azotobacter utilized carbohydrates synthesized by the algae.

Fulton (18) studied the growth patterns of Escherichia coli and Salmonella schottmuelleri in association. He used a synthetic growth medium with aspartic acid as carbon source. Batch units were used, and the growth pattern was followed for seventy-two hours. It was found that the growth cycle of the two organisms in association indicates a tendency for one organism to succeed the other. During the early growth in association, Salmonella schottmuelleri was inhibited to a marked degree, and the population consisted largely of Escherichia coli. After Escherichia coli passed its maximum cell concentration, a luxuriant growth of Salmonella schottmuelleri developed. In the consideration of the growth cycle as a whole, it was noted that there was only a slight inhibition of Salmonella schottmuelleri by Escherichia coli in the association study.

Kessel (19), starting from an observation that Aerobacter aerogenes occurs frequently in the mouths of persons free from dental caries, concluded that Aerobacter aerogenes could inhibit the caries process in teeth by virtue of its ability to produce ammonia. Jeney (20) found

antibiotic effects in D-glutamic acid, D-lysine, and D-arginine. He theorized that the antibiotic effect of one microorganism on another could be due to the fact that one contained exclusively D-amino acids, and the other exclusively L-amino acids.

Charlton (21) used Aerobacter aerogenes and Bacillus subtilis to search for antibiotic substances that would affect the behavior of organisms in mixed cultures. He concluded that inhibition of growth did not occur as a result of direct contact between organisms, nor was it caused by the production of an antibiotic substance. He presented evidence to show that the antagonism was due to competition for gaseous nutrients in the medium.

Wynne (22) tested a strain of Aerobacter aerogenes against twenty-one common species of bacteria, and found it to be antagonistic in varying degrees toward fourteen of them. Filtrates of Aerobacter aerogenes cultures did not exhibit antagonism. Wynne mentioned that the mechanism of antagonistic action was unknown but that it could involve some undefined direct action of living cells. Frankel and Wynne (23) determined that antagonism existed between strains of Gaffkya tetragena and seventeen test organisms. These investigators postulated that direct antagonism was the most feasible explanation for the antibiosis exerted by the microorganisms studied. Additional work was published by Bowling and Wynne (24) where fourteen strains of Aerobacter were tested against seven common bacterial

species, and antagonism in varying degrees was noted in all cases. The antagonistic activity was again attributed to a direct antagonism involving close contact of living Aerobacter cells with cells of the inhibited species. Continuing this series of publications on antagonism, Wynne and Norman (25) reached the conclusion that the direct antagonism theory was inadequate to explain the antagonism noted in their experiments with Aerobacter and Gaffkya. They proposed an indirect antagonistic relationship operating through the production of heat-labile antibiotic substances. Lockhart and Powelson (26) reported on antagonisms of various strains of bacteria, and concluded that the inhibition could not be explained by direct antagonism between living cells. They proposed that the exhaustion of some essential nutrient factor was more likely the probable cause for antagonism.

In regard to microbiological problems of frozen food products, Borgstrom (27) discussed food poisoning and noted that the rich bacterial flora which is generally found in frozen products offered the best protection against infections. Peterson, Black, and Gunderson (28) observed that in frozen macaroni and cheese dinners, tremendous numbers of saprophytic bacteria developed, but only after extended incubation at room temperature. Under those conditions, Staphylococci also multiplied vigorously. Additional work by the same investigators (29) (30) (31) showed that in studies carried out with bacteriological media, with

selected cultures, definite repressive effects were noted on the growth of Staphylococcus populations by a mixture of saprophytic bacteria. It was found that even with elevated temperatures and a heavy inoculum of Staphylococcus, the saprophytic bacteria present always grew better than the staphylococci, so as to render foods containing such mixed populations organoleptically unacceptable before the development of massive numbers of staphylococci. The retarding effect was attributed to growth competition between the saprophytic species and the staphylococci. In addition, it was reported that salt concentrations above 3.5 per cent would inhibit growth of all species, although the saprophytes were inhibited to a larger degree, thus decreasing competition and inducing a predominance shift toward Staphylococcus.

Flippin and Michelson (32) found four Escherichia coli cultures that were antagonistic toward Salmonella in eggs and egg solids. These investigators isolated nineteen microorganisms from various sources that showed varying degrees of inhibition of one or more of the Salmonella species. Since this research was attempting to find a bacterial species that would eliminate Salmonella from eggs, the above nineteen strains were tested and found unsatisfactory because they failed to grow on egg white, failed to ferment glucose, or failed to produce an acceptable product after fermentation. They concluded that a nonpathogenic strain of Escherichia coli, which produced

antagonistic action against contaminating Salmonella during the fermentation process in egg white, could be the answer to the production of a Salmonella-free product. Mickelson and Flippin (33), expanding their work on Salmonella control, found that Escherichia coli strains 6-204-55 and 1673A exhibited a strong inhibition of the growth of Salmonella senftenberg, Salmonella organienburg, and Salmonella typhimurium in egg white.

Oberhofer and Frazier (34) screened sixty-six cultures for their ability to influence the growth of four strains of Staphylococcus aureus, and found that the more consistently inhibitory cultures were Streptococcus faecium, Streptococcus faecalis, a nisin-producing strain of Streptococcus lactis, and various meat lactobacilli. Other cultures were found to be less inhibitory, many were not inhibitory at all, and some were even stimulatory. Iandoto, et al. (65) found that the growth of Staphylococcus aureus was inhibited when grown in association with Streptococcus diacetilactu and other lactic streptococci.

Freter (36) showed that Escherichia coli, Aerobacter aerogenes, and Proteus vulgaris inhibited Shigella flexneri in broth cultures kept in an oxygen-free atmosphere. Inhibition was reversed by aeration or by the addition of glucose to the medium. The hypothesis was advanced that the predominant mechanism of the in vivo and in vitro antagonisms studied was based on competition for fermentable carbon sources in a reduced medium.

Shindala, et al. (37) studied the mixed culture interactions of the yeast Saccharomyces cerevisiae and the bacterium Proteus vulgaris. A chemically-defined medium which supports the growth of Saccharomyces cerevisiae but not of Proteus vulgaris was selected. Steady state mixed population levels were established. An abrupt change in dilution rate initiated an adjustment in the population levels until a new steady state was reached. Several compounds were added to the mixed culture to determine their effect on the dependence of Proteus vulgaris on the yeast. Niacin, niacinamide, and NAD were added, and in all cases the dependence was broken and a competitive situation was created with the faster growing bacterium gaining in numbers while the yeast reduced in numbers until continued pumping diluted the added co-factor and the system was restored to the initial steady state.

Hentges and Fulton (38) found that, in pure cultures, environmental changes had marked effects on the Shigella populations, while Klebsiella populations were not affected except at 44°C. or when aerated. In nonaerated mixed cultures, under different environmental conditions, Klebsiella interfered with the multiplication of Shigella. In aerated mixed cultures, the presence of Klebsiella had no effect on Shigella multiplication.

Guthrie, et al. (39) studied interactions between Escherichia coli and Pseudomonas aeruginosa. It was determined that, when glucose or glycerol was used as carbon

source, no interactions between Escherichia coli and Pseudomonas aeruginosa were noted at any pH tested. It was reported that growth of Pseudomonas aeruginosa was increased when grown in a mixed system using two per cent lactose as carbon source, and that the growth of a pure culture of Pseudomonas aeruginosa was increased if grown on mixed culture filtrates using lactose as carbon source.

Gibson (40) noted pronounced microbial population changes when the pH of the rumen of sheep was abruptly lowered by indigestion induced by a sudden change in diet from hay to grain. The protozoa were killed, there was a very large increase in the proportion of Gram-positive organisms, particularly Streptococcus bovis.

B. Review of Literature in the Bioenvironmental Engineering Field

Activated sludge consists of a flocculent assemblage of microorganisms, organic matter, and inorganic materials. Rich (41) has classified the microorganisms in activated sludge as including bacteria, molds, protozoa, and metazoa, such as rotifers, insect larvae, and worms. Pipes (42) classified the organisms which have significant roles in the activated sludge process as floc-formers, saprophytes, predators, and nuisance organisms, this classification being made in accordance with their roles in connection with waste treatment. Saprophytes were described as the organisms which actually break down the organic matter in the waste, while predators have been described as those

which feed upon the saprophytes. Floc-forming organisms were described as those which form a sludge with good settling characteristics. Nuisance organisms were described as those which interfere with the proper operation of the process when they are present in large numbers.

A considerable amount of research has gone into the determination of the type of microorganisms which are predominant in activated sludge. Unfortunately, the results of the research have not been altogether satisfactory, and this is still an area in which a great deal of research needs to be done. It is not surprising to learn that the efficiency of biological waste treatment has not shown a great deal of improvement in the past when it is realized that the identity of the biological entities involved in waste treatment has not yet been fully determined.

In 1914, Johnson (43) reported on the growth of activated sludge and indicated that it was possible that Zoogloea ramigera, assisted by other minute organisms, chiefly of animal origin, could be responsible for the purification of waters. Winogradsky (44) studied activated sludge and found that the predominant organisms were of the genus Nitrosystis.

Hotchkiss (45) made bacteriological studies of raw sewage and of effluents from Imhoff tanks, filter beds, and settling tanks, and classified the bacteria contained therein in accordance with their physiological activity into proteolytic, sulfur-cycle, and nitrogen-cycle forms.

Butterfield (46) isolated a bacterium, Zoogloea ramigera, from activated sludge and showed that such a bacterium was capable of stabilizing a liquid organic substrate and of producing floc which was characteristic of activated sludge. This work, published in 1934, firmly established the activated sludge treatment as a biological process.

Gilcrease (47) reported that large zoogloeal bacteria were present in sewage and that films of trickling filters contained these as well as filamentous bacteria. With this paper, a tendency was initiated to call floc-forming organisms "zoogloeal," in many cases obscuring and bypassing the issue of whether or not such organisms belong to the species Zoogloea ramigera. Butterfield, et al. (48) emphasized the primary importance of bacteria in the activated sludge process, and showed that bacteria are responsible for the production of activated sludge. These researchers also noted that the primary prerequisites of the purifying organisms in activated sludge were, in addition to oxidizing capacity, the ability to grow in a liquid medium and to form floc.

Butterfield and Wattie (49) isolated pure cultures of the predominant bacteria found in growths on the stones of trickling filters, and found them to be zoogloeal in nature and similar to the predominant bacteria found in activated sludge. Furthermore, they showed that the bacteria isolated from trickling filters produced a floc with the same

general appearance as activated sludge. Heukelekian and Littman (50) made a study of the morphological and cultural characteristics of fourteen cultures of zoogloeal bacteria isolated from as many different activated sludges. These cultures were capable of producing floc and were found to be sufficiently alike to be classed either as one species or as one genus, indistinguishable from the zoogloeal bacterium, Zoogloea ramigera previously described by Butterfield (46). Efforts were also made to isolate other floc-forming organisms, but none was found; it was then concluded that natural activated sludge was comprised mainly of flocculent masses produced by Zoogloea ramigera. Wattie (51) pursued this line of investigation, and classified all floc-forming organisms as members of the species Zoogloea ramigera. As a result of the above described studies, Zoogloea ramigera came to be generally accepted as the predominant bacteria in activated sludge, being responsible for both floc production, and substrate removal.

Ruchhoft, et al. (52) developed two series of experiments, one with normal activated sludge and one with activated sludge developed from a pure culture of bacteria. The experiments were performed using sterilized domestic sewage and synthetic sewage as nutrient sources, and it was observed that there was a remarkable similarity between the purification accomplished by both systems. They concluded that the clarification mechanism was the same for both systems.

McKinney and Horwood (53) found it possible to isolate from the activated sludge organisms other than Zoogloea ramigera which were capable of forming a floc similar to activated sludge when aerated in the presence of a suitable substrate. The floc-forming organisms included Escherichia intermedium, Paracolobactrum aerogenoides, Nocardia actinomorpha, Bacillus cereus, and a bacteriaum belonging to the genus Flavobacterium. Aerobacter aerogenes was found to form a floc of the same nature as the other bacteria, even though it was not isolated from activated sludge. McKinney and Weichlein (54) isolated seventy-two bacteria from samples of activated sludge taken from the San Antonio municipal sewage treatment plant, the Austin biosorption sewage treatment plant, the Celanese Corporation activated sludge plant at Bishop, Texas, and a laboratory unit fed with a completely soluble substrate. Of the strains isolated, twelve were capable of floc formation in sterile settled sewage, and fourteen formed floc in sterile synthetic sewage. All cultures formed floc when incubated, unaerated, in the above noted substrates for seventeen days. Microscopic observation indicated that the floc was the normal result of complete metabolism of the organic substrate, and was not caused by special zoogloeal-producing bacteria. These findings confirmed the results presented by Buswell (43) in 1931, who reported that a pure culture of Bacillus subtilis, incubated in flasks of peptone broth, would produce floc of sufficient density to settle. It is

possible that most, if not all, bacteria can form floc under proper conditions. It is apparent from the data presented by the various investigators previously reviewed that species of bacteria other than Zoogloea ramigera can and do occur in activated sludge, and that it is not necessary to have Zoogloea ramigera present in order to form activated sludge. What is not yet known is the conditions under which predominance may shift toward Zoogloea ramigera, and whether such a shift is a desirable occurrence.

A renewed interest in the role of Zoogloea ramigera is reflected in the work of Dugan and Lundgreen (55). These investigators recognized two species, Zoogloea ramigera and Zoogloea filipendula within the genus Zoogloea, and remarked that both were outstanding producers of zoogloeal masses. They described a procedure for the isolation of Zoogloea ramigera, and indicated that growth of the species was greatly stimulated in chemically defined medium by nucleotides and purine and pyrimidine combinations.

Dias and Bhat (56) reported that Zoogloea and Comamonas were present in significant numbers in samples of activated sludge. They concluded that these two bacteria form the physical basis of the floc particles, and that other bacteria are present as associated organisms. They reported that all organisms isolated by them from activated sludge were capable of forming floc in pure culture, but that Zoogloea and Comamonas did so much more readily.

There has been very little research performed to

systematically clarify the bacterial population in activated sludge. Apparently most of the energy of researchers has been directed toward proving or disproving Zoogloea ramigera as the predominant organism in activated sludge. Hawkes (57) mentioned that some of the earlier investigators found intestinal bacteria predominating. Allen (58) used a homogenizer to disintegrate the flocs, and found that intestinal bacteria were present in negligible numbers. The majority of the strains isolated were members of the genera Achromobacterium, Flavobacterium, and Pseudomonas. He concluded that intestinal bacteria were of little significance in the activated sludge process.

Calaway, et al. (59) presented, in their study of the microbiology and ecology of sewage filtration through sand, the finding that the distribution of the predominant species varied with the filter depth. Fourteen species of common heterotrophic bacteria were isolated and members of the genera Flavobacterium and Bacillus were predominant throughout the filter.

Hamdy, et al. (60) studied the biological treatment of phenolic wastes using a bench scale trickling filter. It was found that operation in this manner would induce a selection of phenol-resistant microflora capable of active reduction of phenolic solutions in concentrations up to 400 mg/l. The three predominating species of bacteria that were responsible for phenol utilization were observed to be a short rod, a coccus, and a spore-forming rod. These

organisms were tentatively identified as Pseudomonas, Micrococcus, and Bacillus.

Jasewicz and Porges (61) performed laboratory investigations into the microbial nature of aerated dairy waste sludge. It was found that under endogenating conditions, members of the genera Alcaligenes, Flavobacterium, Pseudomonas, and Micrococcus were predominant. Analysis of sludge organisms which were actively assimilating substrate gave a totally different picture, with members of the genera Bacillus and Bacterium predominating. McKinney and Weichlein (54) found that members of the following genera were present: Achromobacter, Aerobacter, Alcaligenes, Bacillus, Bacterium, Flavobacterium, Microbacterium, Nocordia, Pseudomonas, and Zoogloea.

Rogovskaya and Lazareva (62) examined seven different types of waste and found predominating organisms to be Bacillus, Bacterium, Microbacterium, Pseudomonas, and Sarcina. Dias and Bhat (56) examined seven sludge samples, two from an activated sludge plant and five others from laboratory cultures, and reported the predominating organisms as belonging to the genera Achromobacter, Aerobacter, Alcaligenes, Bacillus, Corynebacterium, Pseudomonas, Spirillium, and Zoogloea, with Zoogloea and Comamonas being present in significant numbers.

From the data presented by McKinney and Weichlein, Jasewicz and Porges, Rogovskaya and Lazavera, and Dias and Bhat, it can be surmised that Achromobacter, Alcaligenes,

Bacillus, Bacterium, Flavobacterium, Microbacterium, and Pseudomonas are likely to be found in activated sludge. To this list might also be added Zoogloeal ramigera, although it was not reported by either Jasewicz and Porges, or Rogovskoya and Lazareva. Also present, as suggested by Hawkes (56), should be Nitrosomonas and Nitrobacter, particularly if the detention time in the aerator is somewhat long.

In 1927, Morgan and Beck (63) noted severe bulking in the Des Plaines River treatment plant at Chicago. The sewage arriving at the plant was found to contain as high as 10,400 mg/l of glucose. This carbohydrate was later proven to have been present because of the dumping of residues emanating from the illicit fermentation and distillation of corn sugar. Ruchhoft and Watkins (64) identified the filamentous organisms which overran the above plant and classified them as belonging to the genus Sphaerotilus. They concluded that carbohydrate wastes stimulated the growth of undesirable filamentous organisms in activated sludge.

Haseltine (65) found that high air requirements were necessary to avoid bulking and proposed that bulking was caused by the disruption of the balance between adsorption and biological oxidation. He theoreized that overloading causes bulking, and indicated that Sphaerotilus growth may be a secondary cause of sludge bulking. Smit (66) stated that bulking was materially influenced by the presence of carbohydrates. He showed that glucose, sucrose, and

lactose were particularly deleterious, while starch had a much lesser effect. He also pointed out that regardless of the deterioration in the settling characteristics of the sludge, carbohydrates continued to be metabolized with rapidity. Smit also made the observation that, since the carbohydrate content of the sewage in Amsterdam, Holland, was far below the concentration required to induce bulking, it followed that the real cause of bulking was to be found elsewhere.

Smith and Purdy (67) failed to build up a culture of bulking sludge using sterile sewage as substrate. Also, they recommended chlorination of the return sludge as a useful method for combating bulking. Ingols and Heukelekian (68) studied bulking in the light of the known facts that bulking can be induced by the addition of carbohydrates, and that treatment plants bulked in the absence of appreciable quantities of carbohydrates. It was found that when there was a limited supply of nitrogen present, zoogloeal organisms could not completely utilize carbohydrate, leaving an excess to stimulate the growth of Sphaerotilus. It was also found that when an abundance of nitrogen was present, zoogloeal organisms could compete with Sphaerotilus for only a short time, because the latter develops at a higher rate. It was also pointed out that Sphaerotilus, being facultative, could favorably compete with zoogloeal organisms.

The New England Association in one of its sewage

research reviews (69) has noted that no instances of bulking have been reported with carbohydrate-free sewage under laboratory conditions from activated sludge units operated continuously for over twenty-five years at the Lawrence experiment station. It also reported that efforts to induce bulking with starch, sugar, and filamentous organisms had been unsuccessful.

Ruchhoft, et al. (70) stated that the large amount of sludge which developed from sewage containing sugar could be the cause of difficulty in sewage plant operation.

Up to 1940, it was assumed that all bulking was caused by Sphaerotilus. Lackey and Wattie (71) indicated that bulking sludge was usually caused by Sphaerotilus natans, although other filamentous organisms could be responsible for bulking under certain conditions. They also indicated that Bacillus mycoides in pure culture sometimes grew as filaments in such a way that it could be mistaken for Sphaerotilus.

Littman (72) succeeded in producing bulky sludge in two days with a natural activated sludge fed with excessive carbohydrate and limited nitrogen. The predominating bulking organism was identified as Sphaerotilus. Littman also attempted to build up a culture sludge of Sphaerotilus in sterile sewage, but his efforts met with failure.

Heukelekian and Ingols (73) accomplished bulking of activated sludge with sewage by diluting the concentration of oxygen in the air with nitrogen gas. They concluded

that under conditions of oxygen deficiency, Sphaerotilus, which could grow both under aerobic and anaerobic conditions, becomes predominant over Zoogloea, an aerobe. They pointed out that the supply of oxygen should be sufficient not to interfere with the purification mechanism, and yet inadequate for complete aerobic stabilization. Also, it was stated that bulking in the presence of carbohydrate was due to Sphaerotilus but not through oxygen deficiency created by high carbohydrate concentration, rather as a direct specific food response since Sphaerotilus utilizes carbohydrate more efficiently than Zoogloea. Although this paper had great scientific merit, it represents a grave oversimplification of the biological processes involved, since it assumes the existence of only one bulking organism and one floc-forming organism, Zoogloea ramigera.

Ingols and Heukelekian (74) continued their research on bulking, and presented data showing bulking of activated sludge when agitated with a limited amount of oxygen and fed with carbohydrate, calcium butyrate, peptone, glycerol, or calcium propionate. Bloodgood (75) suggested that one of the causes of sludge bulking might be the swelling of the zoogloeal organisms. Ruchhoft and Kachmar (76) showed that Sphaerotilus in pure culture could produce a growth very much like bulking sludge. They worked with a strain of Sphaerotilus natans, which was a strict aerobe, and had an optimum pH range from 6-9. The results indicated that bulking was a response of sludge organisms to a disturbance

in the biological equilibrium, the three primary factors involved being sludge composition, food, and rate of oxygen supply. They did not consider the appearance of Sphaerotilus natans as the primary cause for bulking. Logan and Budd (77) pointed out that bulking would occur not only when a system was overloaded, but also when underloaded. Heukelekian and Weisberg (78) showed that activated sludge could bulk without an overgrowth of filamentous organisms, and indicated that to obtain a Sphaerotilus type of bulking it was necessary to feed sugar.

Ludzack and Schaffer (79) developed activated sludge cultures in the laboratory using a suspension of commercial dog food as substrate, and using a glucose-gelatine solution. Each was studied at temperatures of $5^{o}C$ and $30^{o}C$. They observed bulking sludge with both substrates, and noted that sludge was more likely to develop Sphaerotilus bulking at $5^{o}C$, and concluded that the filamentous organism had a better competitive position at the lower temperature. Dondero, et al. (80) pointed out that the best isolation method for Sphaerotilus was quite different from the method used for bacterial surveys on activated sludge, and that it was likely that Sphaerotilus could be isolated from almost any sludge sample if the proper techniques were used. Various isolation techniques were presented in the paper.

The role of fungus in the production of bulking sludge is not altogether clear. Genetelli and Heukelekian (81) have reported on fungus causing bulking in a laboratory

activated sludge culture. No identification of the fungus was made. These investigators proposed that both Sphaerotilus and fungus types of bulking could be induced at the same loading with glucose by varying the buffering capacity of the system. Lackey (82) suggested that fungi could play an important part in the formation of floc in activated sludge. Cooke (83) included samples from several activated sludge plants in his survey of fungi in polluted waters. It was reported that members of the general Cladosporium, Margarinomyces, Aureobasidium, Geotrichum, and Trichoderma were present in large numbers in activated sludge. The presence of fungi that could produce bulking in activated sludge was thereby noted.

Hawkes (56) states that fungi have not been extensively reported in connection with activated sludge, but that this may be due to lack of research rather than lack of fungi. He also reported that Geotrichum has caused bulking problems at the sewage plant at Yardley, England. Cooke and Ludzack (84) reported that the rotifer-trapping fungus Zoophagus indidians caused bulking in a laboratory activated sludge unit.

The difficulties of classifying and identifying bulking organisms from activated sludge have been clearly demonstrated by Pipes and Jones (85). These investigators worked with Geotrichum for over a year under the impression that it was Sphaerotilus. In view of the above experience, it is difficult to give credence to the various reports of

bulking in which the authors have classified the predominating organism as Sphaerotilus without having shown positive evidence of its presence. This is particularly evidenced by the widespread belief in the water pollution control field that Sphaerotilus growth would occur at low pH, when it has been conclusively demonstrated (76) that the optimum pH range of Sphaerotilus is from six to nine, and that this genus is very sensitive to pH values below five. There is also some doubt as to the soundness of classifying all filamentous organisms causing bulking as Sphaerotilus, since there is substantial evidence (66)(56)(71)(53) that other organisms, e.g., fungi, also can be responsible.

The predominance and role of yeasts in activated sludge is a relatively unexplored area. Cooke, et al (86) reported on yeasts isolated from the same samples used in Cooke's previous paper on fungi (83). In the later work (86) it was found that the genera Candida, Rhodotorula, Turulopsis, and Trichosporon were present in samples taken from activated sludge. These authors did not elaborate on the possible role of the yeasts in the overall activated sludge process.

The role of protozoa in activated sludge was studied by Purdy and Butterfield (87). They reported that a culture containing both bacteria and protozoa would show first a bacterial population increase and then a decrease in number coincidental with a rapid increase in the protozoa. Pillai

and Subrahmanyan (88)(89) proposed that protozoa were predominant in activated sludge, at least to the point of being mainly responsible for sewage purification. This concept was thereafter disproved by various investigators, among them Heukelekian and Gurbaxani (90). Bhatla and Gaudy (91) in a classical paper explained the role of protozoa, and their interaction with bacteria in the diphasic exertion of BOD.

Jeris and McCarty (92) studied anaerobic digesters and suggested that digester upsets may occur due to a change in predominance of the acid-forming bacteria, resulting in the accumulation of different substrates for which the appropriate species of methane bacteria were not present.

Gaudy (93) experimented with four organisms, Alcaligenes faecalis, Pseudomonas fluorescens, Serratia marcescens, and Flavobacterium esteroaromaticum. He found that a mixture of organisms proved to be more efficient in substrate utilization than the pure cultures. Also, from the data collected, he concluded that with the four organisms studied, it was not always possible to predict predominance in mixed populations by pure culture studies, since such studies gave no indication of the interplay between the species when grown together.

Engelbrecht and McKinney (94) studied acclimated sludge cultures, and concluded that the chemical structure of the substrate fed was the controlling factor in the predominance of microorganisms when all environmental factors

were constant. They also concluded that activated sludges developed on strictly related chemical compounds had similar morphological appearance and produced similar biochemical changes. The conclusions stated in this paper were simple, straightforward, and logical, and would have represented a tremendous advance to our field if they had been borne out by factual observation. Unfortunately, they were not. Rao and Gaudy (95) conducted long-term studies with heterogeneous populations, and found that for sludge samples taken at different times, the relationship between initial solids and COD removal varies for a single substrate. Also, it was reported that the cell yield varied for a single substrate. Both of these observations were explained by observation of concurrent changes in predominance in the system studied, thereby indicating that prediction of predominating organisms solely on the basis of the structural configuration of the substrate may not be a valid concept.

Leal (96) studied interaction of bacterial systems under controlled environmental conditions and concluded that cell concentration and the mode of predominance in mixed cultures could not be quantitatively predicted based on studies with pure cultures. Kincannon and Gaudy (97) studied the effects of high salt concentrations on activated sludge, and concluded that acclimation of sludges to high salt concentrations involved a selection of species rather than an acclimation of all the species present.

Gaudy, et al. (98) studied the occurrence of the plateau in BOD exertion, and formulated four theories explaining the existence of the plateau. One of the theories was that the plateau was caused by a change in predominance that would occur in a heterogeneous system after the exogenous substrate had been exhausted.

Cassell, Sulzer, and Lamb (99) studied continuous flow systems developed from natural sewage populations using skim milk as substrate. They observed that all parameters which reflected biological activity fluctuated continuously, and concluded that the fluctuations were the result of microbial interactions. They succeeded in establishing different predominating populations at high and low detention times. They also concluded that when two mixed populations competed and neither had great selective advantage, the mixed culture would be unstable.

The short analysis of the literature which has been presented serves to emphasize the many areas of biological science in which species predominance is of considerable interest. Also noted is the deficiency of basic work that has been published dealing with causes and effects of changes in predominance. No doubt, much more research work in this area needs to be done and should be done to allow scientists to predict and control microbial predominance patterns in the many different situations in which such control would be beneficial.

CHAPTER III

THEORETICAL PRINCIPLES

A. General

The theoretical principles included in this chapter are as follows: theory of enzyme kinetics, bacterial growth kinetics, theory of microbial batch kinetics, and steady state kinetics without recirculation. These theories have been included as a necessary background for the kinetic considerations as applied to predominance patterns in continuous and discontinuous systems, and for the use of kinetic growth constants in studying and predicting relative bacterial population densities.

B. Theory of Enzyme Kinetics

A discussion of classical enzyme kinetics is included here because microbial growth kinetics are based essentially on kinetic formulations for enzyme systems. Microbial growth can be considered to be the result of a large number of enzyme reactions, and as such, the overall reaction rate could be considered to be that of the rate-limiting step.

In an enzyme reaction, it is assumed that an intermediate enzyme-substrate complex is formed. The following

reaction may then be written:

$$[E] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_3} [E] + [P] \qquad (1)$$

where

$[E]$ = concentration of enzyme; $[P]$ = product

$[S]$ = substrate concentration

$[ES]$ = enzyme-substrate complex

k_1, k_2, k_3 = velocity constants

The rate of formation of ES is

$$\frac{d[ES]}{dt} = k_1([E] - [ES])[S] \qquad (2)$$

where $[E] - [ES]$ is the concentration of the uncombined enzyme.

The rate of disappearnce of ES is

$$-\frac{d[ES]}{dt} = k_2[ES] + k_3[ES] \qquad (3)$$

At steady-state, the rates of formation and disappearance of ES are equal:

$$k_1([E] - [ES])[S] = k_2[ES] + k_3[ES] \qquad (4)$$

Rearranging

$$\frac{[S]([E] - [ES])}{[ES]} = \frac{k_2 + k_3}{k_1} \qquad (5)$$

$$K_m = \frac{k_2 + k_3}{k_1} \qquad (6)$$

where K_m is the Michaelis constant. From Equation (5)

$$[ES] = \frac{[E][S]}{K_m + [S]} \qquad (7)$$

The initial velocity of the reaction, V, is given by the expression:

$$V = k_3 \left[ES\right] \tag{8}$$

When $\left[S\right]$ is made so high in relation to $\left[E\right]$ that all of the enzyme is present as ES, the velocity of the reaction is V_{max}.

$$V_{max} = k_3 \left[E\right] \tag{9}$$

By substituting for $\left[ES\right]$ in Equation (6), and substituting the value of V_{max} given in Equation (8), the Michaelis-Menten equation is obtained:

$$V = \frac{V_{max} \left[S\right]}{K_m + \left[S\right]} \tag{10}$$

From Equation (9) it is seen that when $V = 1/2\ V_{max}$, K_m is equal to the concentration of the substrate that gives half of V_{max}.

Lineweaver and Burk (100) proposed that Equation (10) be inverted and factored to obtain the following equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{\left[S\right]} + \frac{1}{V_{max}} \tag{11}$$

Since the equation for a straight line is

$$y = ax + b$$

it is possible to plot Equation (11) using 1/S and 1/V axes and obtain a straight line where the line cuts the vertical axis (1/V) at a point which gives $1/V_{max}$ and has a slope of K_m/V_{max}.

C. Relationship between Bacterial Growth Rate and Substrate Concentration

The relationships which govern bacterial growth kinetics have been formulated by Monod (101), who proposed the following equation:

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{12}$$

where μ is the growth rate, μ_{max} is the maximum growth rate, S is the concentration of the limiting growth factor, and K_S is a saturation constant numerically equal to the substrate concentration at which the growth rate is 1/2 of the maximum value.

The Monod equation is of an empirical nature. It is, however, to be noted that the equation is similar to the Michaelis-Menten equation for enzymatic reactions. In the light of such similitude, the Monod equation could be considered to be that of the rate-limiting reaction in the conglomerate of enzymatic reactions that constitute bacterial growth.

In this report, the Lineweaver-Burk plot of the Monod equation and the kinetic growth constants derived therefrom have been determined for each pure culture and used to study and predict predominance patterns in mixed cultures.

D. Microbial Batch Kinetics

In the log growth phase, bacterial growth can be represented by the following equation:

$$\frac{dx}{dt} = \mu x \tag{13}$$

where x is the concentration of organisms and μ is the exponential growth rate.

Rearranging and solving:

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{d \ln x}{dt} \tag{14}$$

Equation (14) is the exponential growth equation.

The specific growth rate is related to the generation time, λ, by:

$$\mu = \frac{\ln 2}{\lambda} \tag{15}$$

Solving:

$$\lambda = \frac{0.693}{\mu} = \frac{0.301}{\beta} \tag{16}$$

where β is μ for common logarithms.

E. Steady State Kinetics without Recirculation

In a continuous flow reactor without recirculation, the kinetics of the process can be formulated by performing a materials balance for cells and substrate.

In a materials balance for cells:

$$\begin{bmatrix}\text{rate of cell} \\ \text{increase}\end{bmatrix} = \begin{bmatrix}\text{rate of} \\ \text{growth}\end{bmatrix} - \begin{bmatrix}\text{rate of} \\ \text{outflow}\end{bmatrix}$$

$$\frac{dx}{dt} = \mu x - Dx \tag{17}$$

where D is the dilution rate, i.e., the reciprocal of the detention time.

Equation (17) can be expressed in the following form:

$$\frac{dx}{dt} = x\left(\frac{\mu_{max} S}{K_s + S}\right) - D x \tag{18}$$

by substituting the μ value from the Monod Equation (12).

In a materials balance for substrate:

$$\begin{bmatrix}\text{rate of increase}\\ \text{of substrate}\end{bmatrix} = \begin{bmatrix}\text{rate of}\\ \text{input}\end{bmatrix} - \begin{bmatrix}\text{rate of}\\ \text{output}\end{bmatrix} - \begin{bmatrix}\text{rate of substrate}\\ \text{consumption}\end{bmatrix}$$

$$\frac{dS}{dt} = DS_R - DS - \mu \frac{x}{Y} \tag{19}$$

where S_R is the substrate concentration of the inflow, S the substrate concentration of the effluent, and Y the cell yield.

Equation (19) can also be expressed as:

$$\frac{dS}{dt} = D\,S_R - D\,S - \left(\frac{x}{Y}\right)\left(\frac{\mu_{max}\,S}{K_S + S}\right) \tag{20}$$

by substituting the Monod equation for μ into Equation (19).

In the steady state

$$\frac{dx}{dt} = 0 \tag{21}$$

and

$$\frac{dS}{dt} = 0 \tag{22}$$

Since, in the steady state, $\frac{dx}{dt}$ and $\frac{dS}{dt} = 0$, the steady state equations can be derived from the equations given above.

From Equation (17), when $\frac{dx}{dt} = 0$

$$\mu = D \tag{23}$$

From Equation (19) when $\frac{dS}{dt} = 0$

$$D\,S + \frac{x}{Y}\mu = DS_R \tag{24}$$

Substituting (23) into (24) and rearranging

$$x = Y\,(S_R - S) \tag{25}$$

Equation (25) indicates that the cell concentration in the reactor depends upon yield and the incoming and outgoing

substrate concentration.

Substituting Equation (12) into Equation (23) and solving for S,

$$S = K_S \left(\frac{D}{\mu_{max} - D} \right) \tag{26}$$

Equation (26) indicates that the substrate concentration in the effluent depends upon the dilution rate and the growth rate constants.

An examination of Equations (25) and (26) reveals that when Y, μ_{max} and K_S are known, the concentrations of cells and substrate in a reactor can be predicted for different dilution rates, at a given inflowing substrate concentration.

CHAPTER IV

MATERIALS AND METHODS

A. Description of Equipment

1. Batch Runs

In batch experiments, flasks of special design were used to measure growth rates. These flasks were of 250 ml capacity. Matched test tubes were attached to the flasks by ground glass joints to facilitate making optical density readings. Optical densities were measured by inverting the flasks and placing the test tubes into a spectrophotometer. The flasks, each containing 60 ml of fluid, were incubated on a water bath shaker (Research Specialties Company) maintained at 25°C and operated at a constant speed of ninety strokes per minute. The optical density flasks used are shown in Figure 1.

All optical densities were read using a Bausch and Lomb Model Spectronic 20 spectrophotometer. All absorbancy readings pertaining to growth rate measurements were made at a wave length of 540 mμ .

2. Continuous Flow Experiments

The equipment used in continuous flow experiments is shown in Figure 2. It consisted of a growth medium

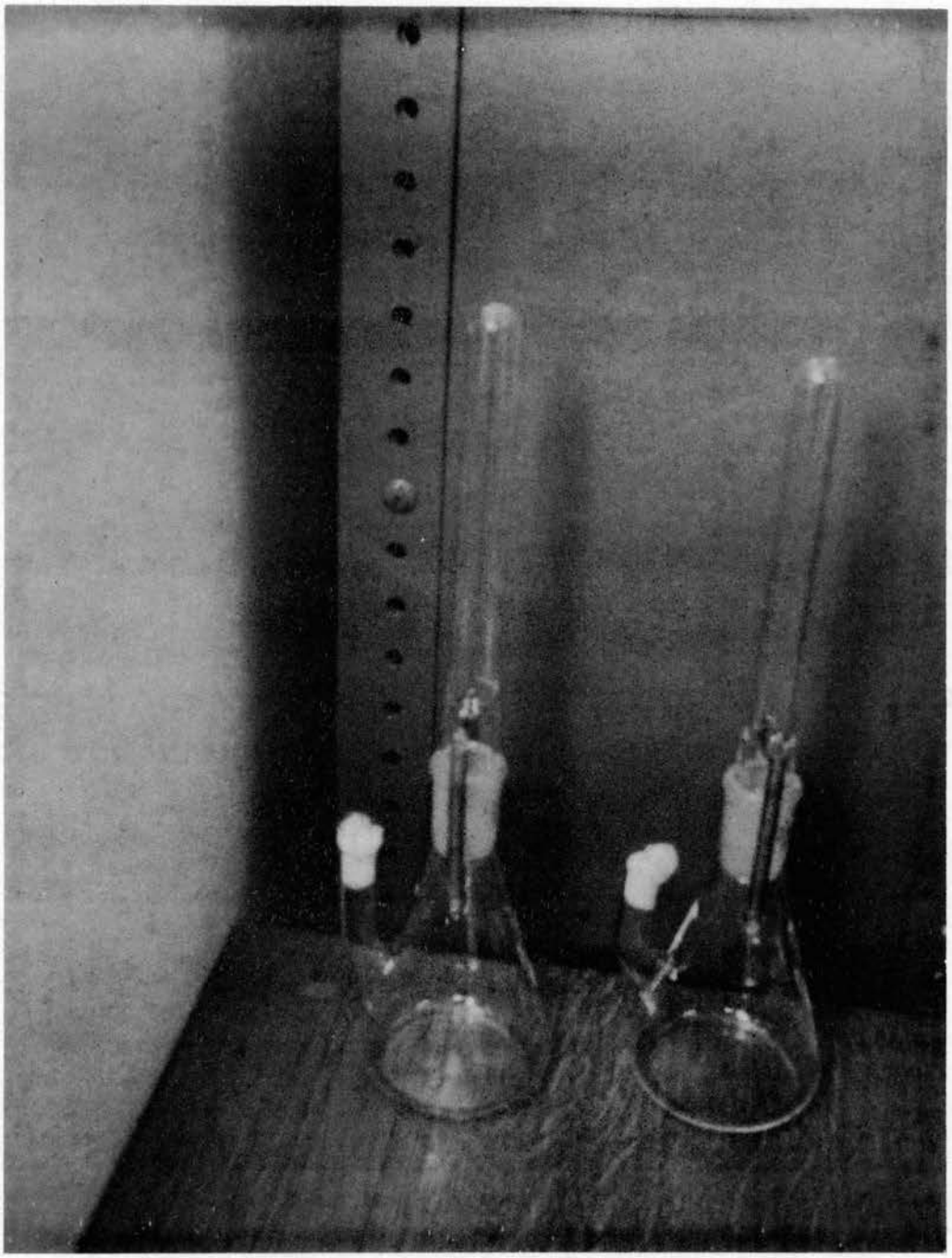

Figure 1 - Flasks used for optical density measurements in batch experiments.

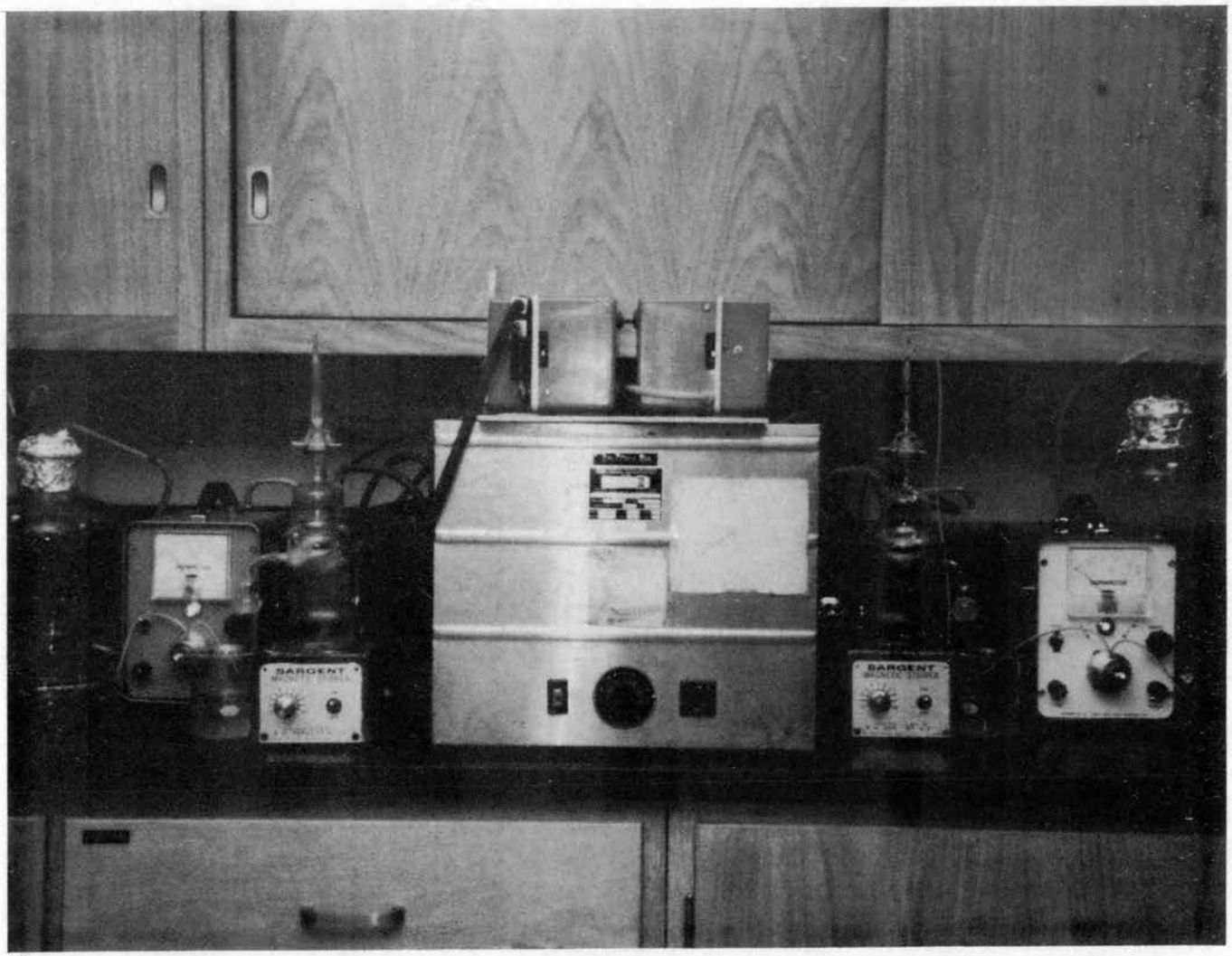

Figure 2 - Continuous flow reactors.

reservoir, a continuous flow reactor (the chemostat), two air filters, an air flow meter, a water bath, a pump for pumping water through the water jacket of the chemostat (Beckman Model 746 solution metering pump) and a low flow pump (Sigmamotor, Inc. AL-2-E) to introduce growth medium into the reactor.

In several of the experiments the chemostat was placed over a magnetic stirrer, and a small magnet was placed inside. The magnet was then rotated at a constant speed to increase the mixing inside the reactor.

B. Synthetic Waste

The synthetic waste used in the experiments consisted of a carbon source and the salts listed in Table I.

TABLE I

CONSTITUENTS OF A SYNTHETIC WASTE

$(NH_4)_2SO_4$	500 mg/l
$MgSO_4 \cdot 7H_2O$	100 mg/l
$MnSO_4 \cdot H_2O$	10 mg/l
$FeCl_3 \cdot 6H_2O$	0.5 mg/l
$CaCl_2$	7.5 mg/l
KH_2PO_4	526 mg/l
K_2HPO_4	1070 mg/l
Tap Water	100 ml/l
Distilled Water	to volume

C. Analytical Techniques

1. Chemical Oxygen Demand

The COD of the samples was determined in accordance with the procedures indicated in "Standard Methods for the Examination of Water and Wastewater" (102)(103). Silver sulfate was used in all determinations as a catalyst.

2. Biological Solids

The concentration of biological solids was determined by the membrane filter technique (HA, 0.45μ filter manufactured by the Millipore Filter Co.). The filters were placed on alumination dishes, oven-dried at 103^{o}C for two hours and placed in a desiccator for at least two hours before tare weight was determined. Measured samples were filtered through tared filters under vacuum, submitted to the same drying procedures and weighed. From the difference in weight, the concentration of biological solids was determined.

3. Oxygen Uptake

The oxygen uptake of the samples was determined using a Warburg respirometer (Gibson Medical Electronics) operated at 25^{o}C with a standard oscillating velocity of 110 strokes/min. Forty ml samples were used in all experiments.

4. Glucose

Glucose determination was made by the Glucostat test in accordance with the procedures outlined in the Glucostat Manual of the Worthington Biochemical Corporation (104).

5. Sorbitol

Sorbitol determinations were made using periodate oxidation, in accordance with the following procedure, which has been previously used by Komolrit (105):

a. Place an aliquot containing 0.05-0.3 mg sorbitol in a test tube.

b. Make up the sample to 2.0 ml with distilled water.

c. Add 0.1 ml of 10N H_2SO_4.

d. Add 0.5 ml of 0.1 M periodic acid.

e. Allow five minutes to react, and add 0.5 ml of 0.1M sodium arsenite. Mix, and wait 10-5 minutes.

f. Make up volume to 10 ml by adding 6.9 ml of absolute alcohol.

g. Take 1 ml of the above solution, place in a test tube and add 10 ml of chromotropic acid solution.

h. Heat in a boiling water bath for 30 minutes under diffused light.

i. Cool to room temperature, and read optical density at a wave length of 570 mμ .

The chromotropic acid reagent is prepared by dissolving 1 gm of the chemical in 100 ml of distilled water; the solution is then filtered. A sulfuric acid solution is prepared by adding 300 ml of concentrated H_2SO_4 to 150 ml of distilled water. This solution is cooled and added to the first solution to make up a volume of 500 ml. The resulting reagent is then stored in a brown bottle.

When the sorbitol determination is performed, at least

two blanks and several sets of standards are run concurrently with the unknown samples.

6. Sucrose

Sucrose determinations were performed by the resorcinol method indicated by Roe (106). The procedure is as follows:

a. The resorcinol reagent is prepared by dissolving 0.1 gm of resorcinol and 0.25 gm of thiourea in 100 ml of glacial acetic acid.

b. The acid solution is prepared by mixing five parts of concentrated HCl with one part of distilled water.

c. Two ml of a sample containing from 0.04 to 0.16 mg of sucrose are placed in a test tube.

d. Add 1 ml of resorcinol reagent.

e. Add 7 ml of HCl solution.

f. Heat in a water bath at 80°C for ten minutes.

g. Read at a wave length of 520 mμ after thirty minutes of heating.

7. Volatile Acid Determinations

Volatile acids were determined using a Model 810 gas chromatograph (F & M Scientific Company). A 1/4" glass column packed with Polypak-2 (Hewlett-Packard Co., Avondale, Pa.) was used. The carrier gas utilized was helium. Hydrogen was supplied at 20 psi, and air at 33 psi. Helium was supplied at the rate of 100 ml/min at 60 psi. The detector temperature was 275°C, the injector temperature was 300°C, and the oven temperature was 200°C.

D. Bacteriological Techniques

1. Sterile Techniques

All media, glassware and equipment used in contact with bacterial cultures were sterilized for fifteen to sixty minutes at 15 psi and 121°C in an autoclave, or at 160°C for no less than two hours in an oven.

2. Plate Counts

The enumeration and differentiation of bacteria was accomplished using the spread plate surface counting technique. Difco nutrient agar with 0.5% Bacto agar added was used in all cases. After proper dilution of a sample, 0.1 ml was placed upon the agar surface in a petri dish. The dish was placed on a turntable, and the sample was spread evenly over the agar surface by rotating the turntable while holding a curved glass rod on the surface. The glass rod was sterilized by immersion in ethyl alcohol or isoprophl alcohol followed by flaming.

In all cases, duplicate plates were prepared. These were counted, using Quebec Colony counter, after being incubated at 25°C.

E. Bacterial Cultures

The pure cultures of bacteria used in these studies were either isolated from sewage or known to be present in sewage. These organisms were selected for study because their growth characteristics when placed on an agar surface were such as to allow rapid enumeration and accurate

identification. The organisms used were as follows:

Pseudomonas aeruginosa

Serratia marcescens

Escherichia coli, strain K-12

An organism tentatively identified as Escherichia intermedia

An unidentified organism, hereafter called Yellow organism

An unidentified organism, hereafter called Blue organism.

F. Experimental Protocol

1. Batch Studies

Bacterial cultures were maintained (at a constant temperature of 25°C) in shake flasks (90 strokes/min) containing 60 ml of medium. Every four to five days the cultures were transplanted by removing 5 ml to another flask containing 55 ml of growth medium which contained sufficient substrate to yield 1000 mg/l of carbon source when the transfer was accomplished. Culture purity was checked after every transfer by making streak plates. Approximately eighteen hours before each experiment, transfers were made of each of the cultures to be utilized, in accordance with the procedure outlined above, and the seed used in the experiment was taken from these flasks. In all of the culture transfers as well as in all of the experiments performed in connection with this research, sterile techniques were scrupulously observed.

2. Kinetic Growth Rate Studies

The kinetic growth rate studies were carried out by pipetting two to four ml of the seed culture into the optical density flasks containing sufficient salts medium, buffer, and substrate for a final volume of 60 ml. These flasks contained various substrate concentrations from 100 mg/l to 1000 mg/l. Duplicate flasks for each substrate concentration were used. The optical density of each suspension was read immediately after inoculation, and the flasks were placed in the water bath shaker which was maintained at a constant temperature of 25°C. Optical density readings were taken at hourly intervals until no additional change in absorbancy was observed.

3. Growth Rate Comparison Experiments

The growth rate comparison experiments were performed in a manner similar to the growth rate studies described above. Six optical density flasks were prepared, two containing one substrate, two containing a second substrate, and the remaining, a mixture of both substrates. Three of the shaker flasks (one of each duplicate set) were seeded with the organism acclimated to one substrate, while the other three flasks were seeded with the same organism acclimated to the second substrate. Optical densities were then read, and the change in absorbancy was followed for each flask throughout the growth cycle.

4. Studies on Biochemical Behavior

For the biochemical behavior experiments, a large

container of the salt medium-buffer-substrate mixture was seeded with the culture to be studied. Sixty ml portions of the culture were transferred to sterile 250 ml Ehrlenmeyer flasks and placed in the shaker. Forty ml portions were added to sterile Warburg flasks, and 60 ml portions were added to optical density flasks. The oxygen uptake was followed with the Warburg apparatus and samples were collected periodically from one of the flasks for plate counts. Flasks were removed from the shaker every one to two hours and the contents filtered and analyzed for COD and specific substrate. Similar procedures were followed with two or three organisms in mixed culture experiments. Experiments on sequential substrate removal were also performed in this manner, with two substrates being added to the culture.

5. Starvation Experiments

The starvation experiments were carried out by preparing two cultures of bacteria grown on glucose in the manner already described. After inoculation, twenty-four to thirty-six hours were allowed until it became certain that all substrate was exhausted and the cultures were in the endogenous phase. Thirty ml of each of the "endogenating" cultures were placed on the shaker at 25^{o}C, and samples were removed each twelve to twenty-four hours for plate counts. For the control systems, the procedure was the same except that the cultures were not mixed.

6. Continuous Flow Studies

In the continuous flow studies, after the entire

assembly had been properly sterilized, the chemostat was inoculated and the cells allowed to grow for twenty-four hours in batch culture before a feed flow rate was established. Aeration was provided at a flow rate of 1000 cc of air per minute. After a feeding flow rate was set, the chemostat was allowed to equilibrate through at least three detention times. After that time, effluent samples were checked for steady-state conditions by means of optical density. After such conditions were achieved, as shown by three consecutive samples having identical optical density, sampling was begun by collecting the required volume for analysis in a tube placed in an ice bath. These samples were filtered through a Millipore filter; the filtrate was then analyzed for substrate and volatile acids. To assay for viable population, a one ml sample was collected directly from the reactor by means of a sterile pipette. These determinations also served to check the purity of the aerating culture.

CHAPTER V

RESULTS

A. General

The results herein presented cover a very broad area in which some topics may appear somewhat unrelated. This situation arises because of the essentially exploratory nature of the work which necessitated involvement in a more diversified effort than would be justified by research in a more narrow or specific area. The results could have been presented in several different ways; however, the order chosen appears to the writer to provide for a unified and easily followed report. The results are presented in five general sections:

1. Batch studies with pure cultures
2. Batch studies with mixed cultures
3. Continuous flow studies with pure cultures
4. Continuous flow studies with mixed cultures
5. Species predominance control experiments.

A presentation of the results of each particular subsection is followed by an analysis of the results of the subsection. It was felt that this mode of presentation would allow a more unified discussion of the research

findings in the general discussion of results in Chapter VI.

B. Batch Studies with Pure Cultures

1. Kinetic Growth Rate Studies using Glucose as Substrate

The investigation of the kinetic growth rate constants for each pure culture studied was the basic starting point in this work. Figure 3 shows the growth rate curves of the Blue organism at different initial glucose concentrations. As expected, a greater total growth resulted from higher substrate levels. The same results plotted on semi-log paper are shown in Figure 4. The slope of the straight line portion of the semi-log plots represents the logarithmic growth rate and is used to calculate the specific growth rate, μ. The average value of the specific growth rate at each substrate concentration computed from at least two experiments similar to the one shown in Figures 3 and 4 are plotted against substrate concentrations in Figure 5. This figure represents a Monod plot which permits the graphical determination of the kinetic growth rate constants. Values of μ_{max} and K_s for the Blue organism are shown in Figure 5.

Figure 6 shows an arithmetic plot of optical density vs. time for the organism Pseudomonas aeruginosa. The same results plotted on semi-log paper are shown in Figure 7. The plot of specific growth rate vs. substrate concentration is shown in Figure 8. It is noted that all of these curves bear a close relationship to those of the Blue organism, although the calculated μ_{max} is somewhat lower and the K_s higher than that of the Blue organism.

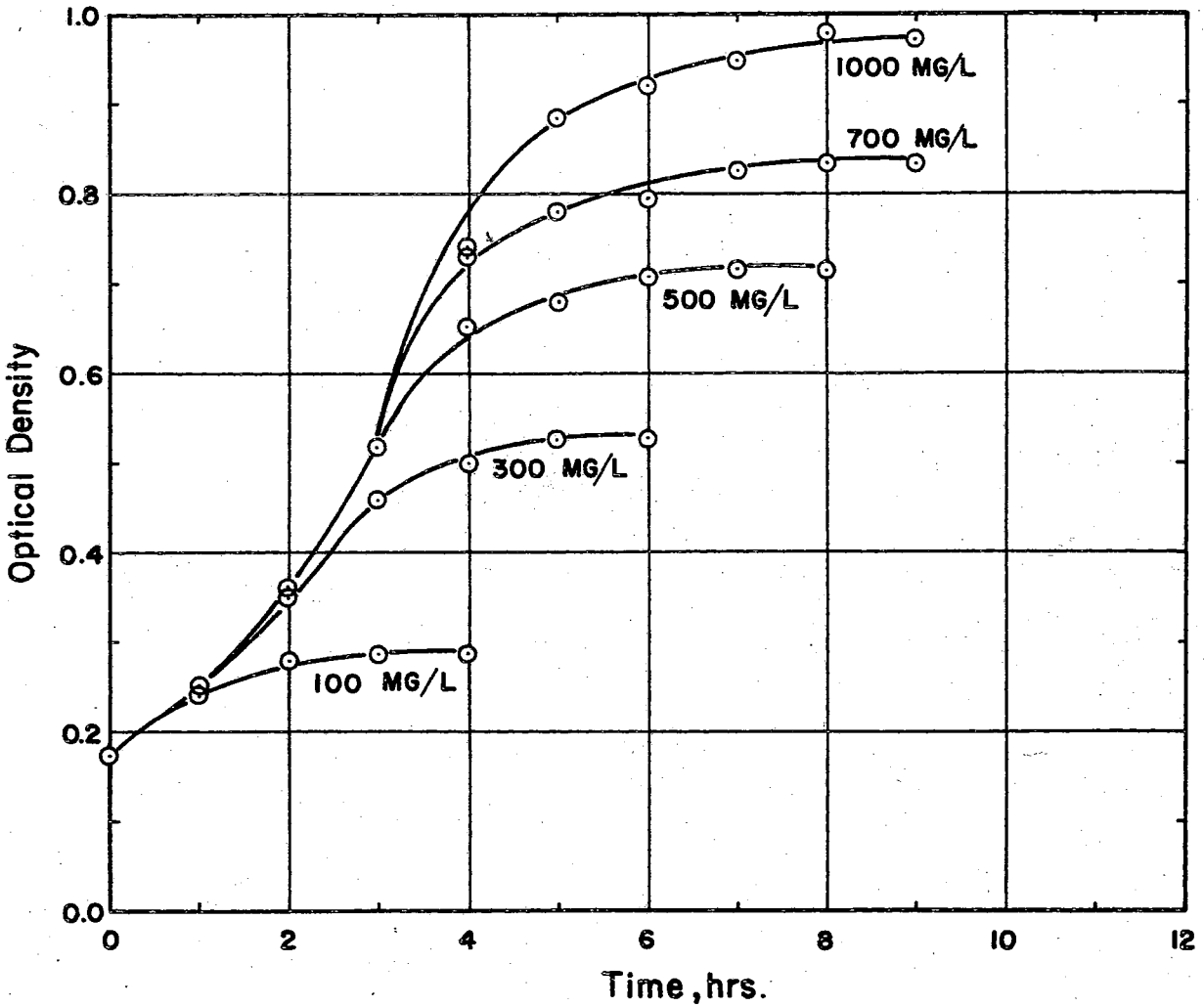

Figure 3. Growth of Blue organism on various concentrations of glucose.

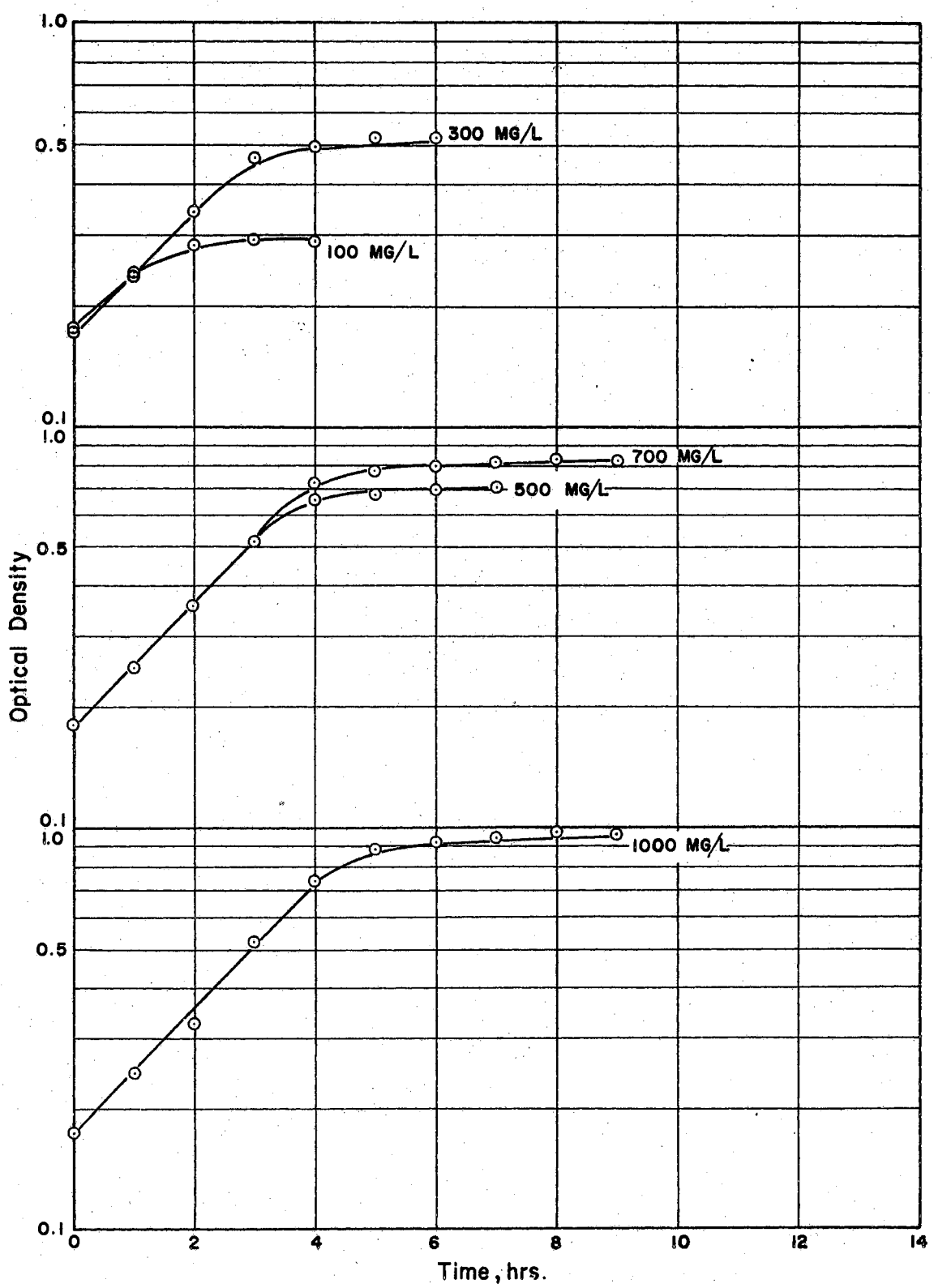

Figure 4. Effects of glucose concentration on growth rate of Blue organism.

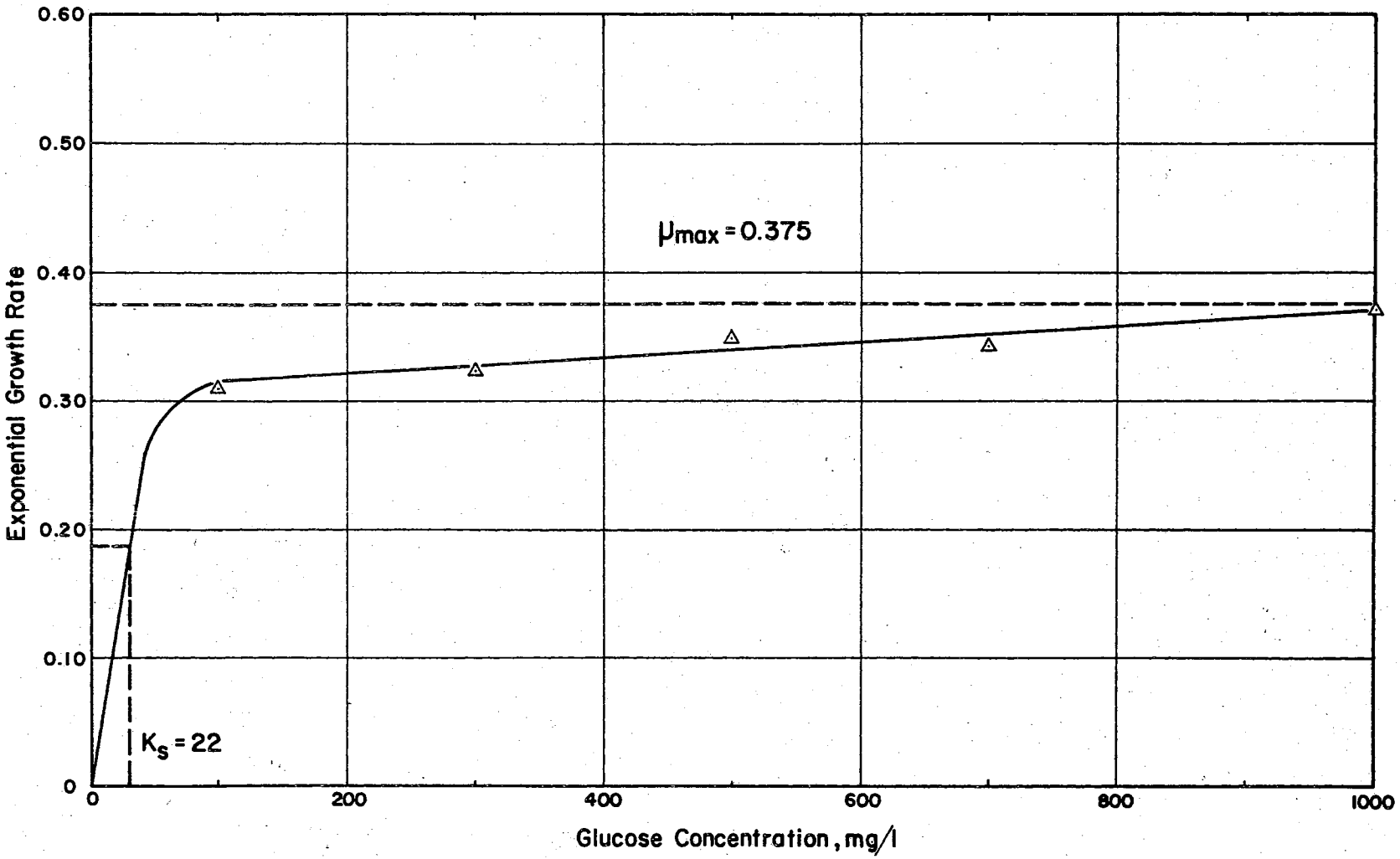

Figure 5. Determination of maximum growth rate (μ_{max}) and saturation constant (K_s) for Blue organism.

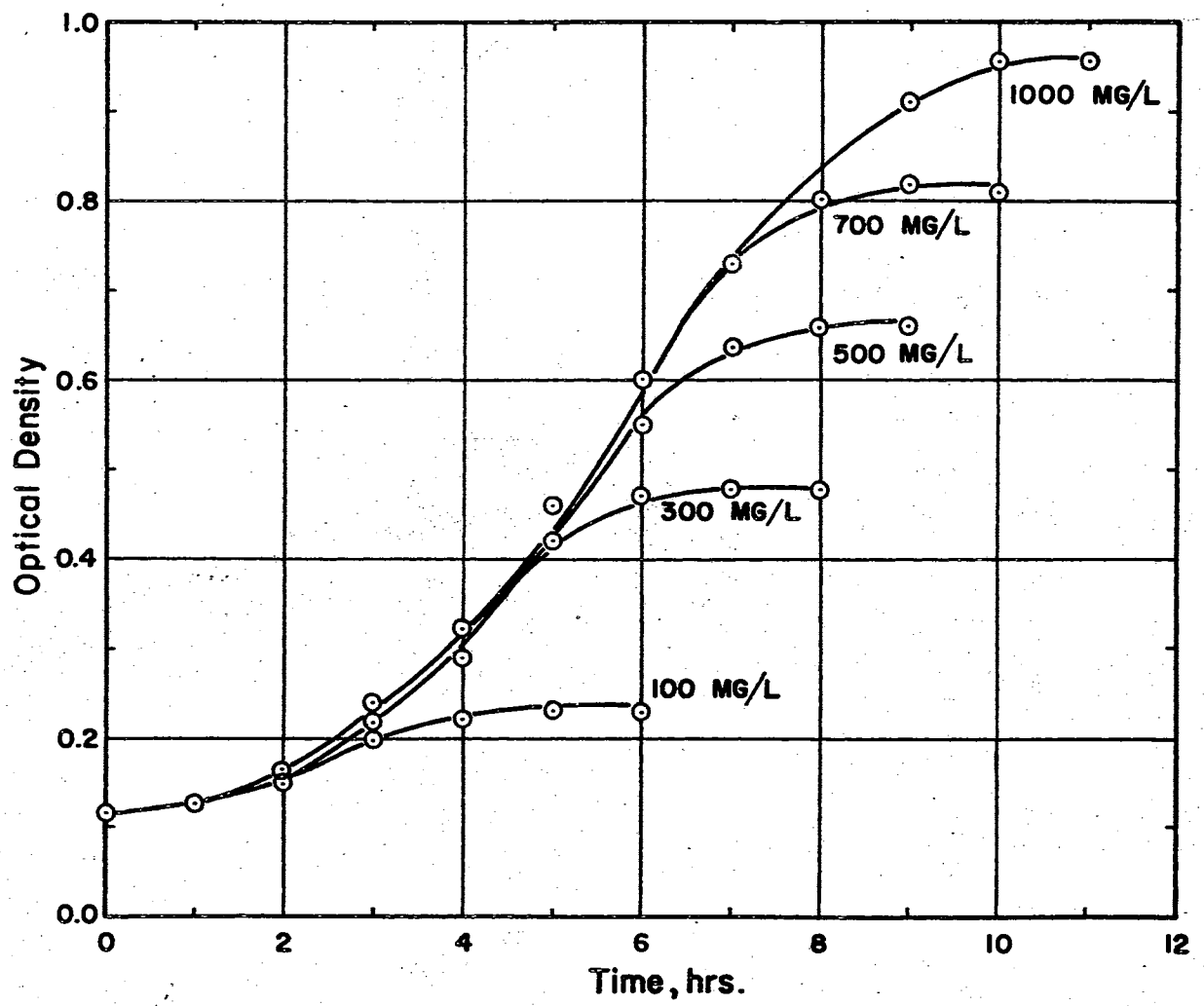

Figure 6. Growth of Pseudomonas aeruginosa on various concentrations of glucose.

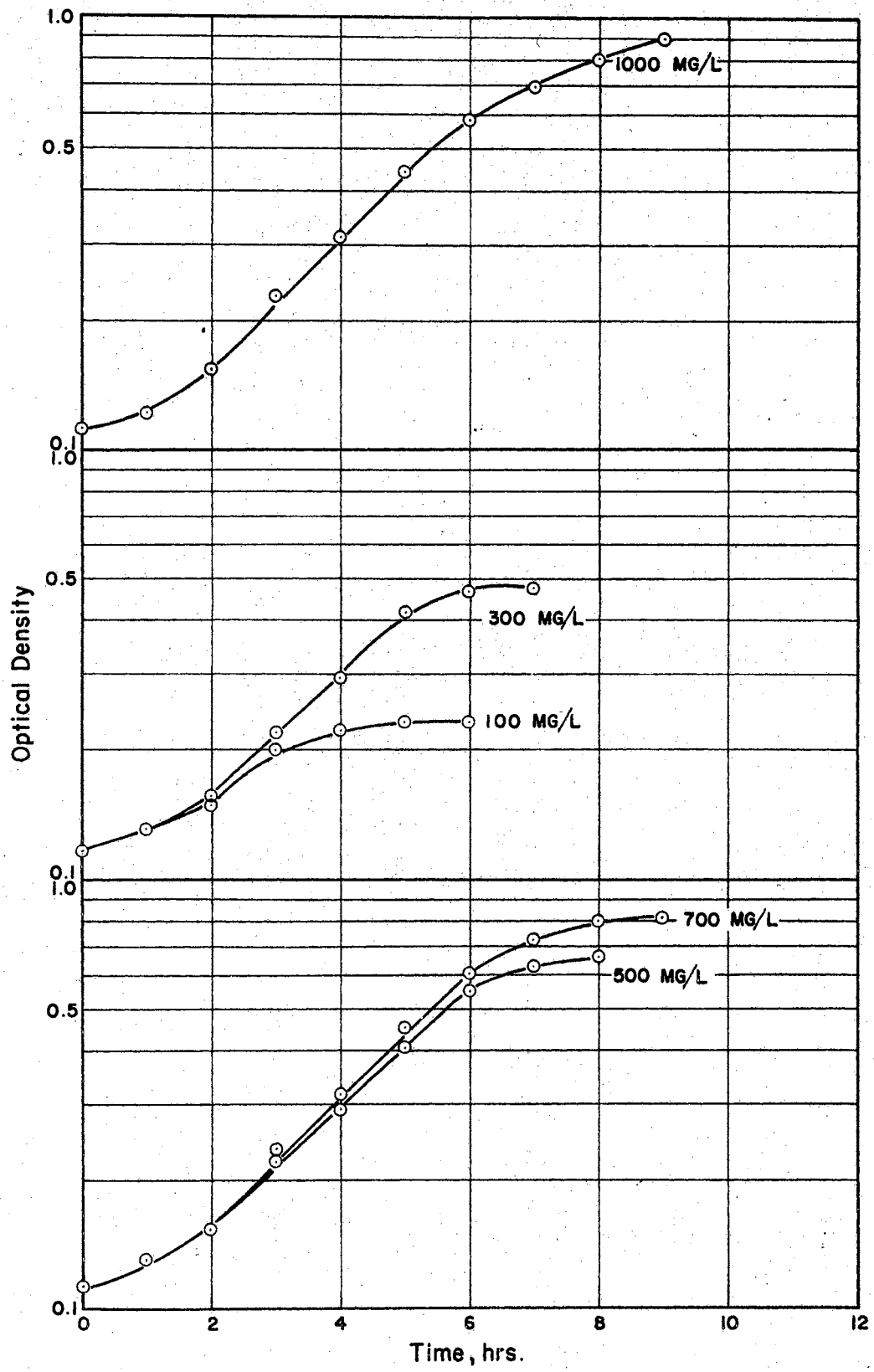

Figure 7. Effects of glucose concentration on growth rate of Pseudomonas aeruginosa.

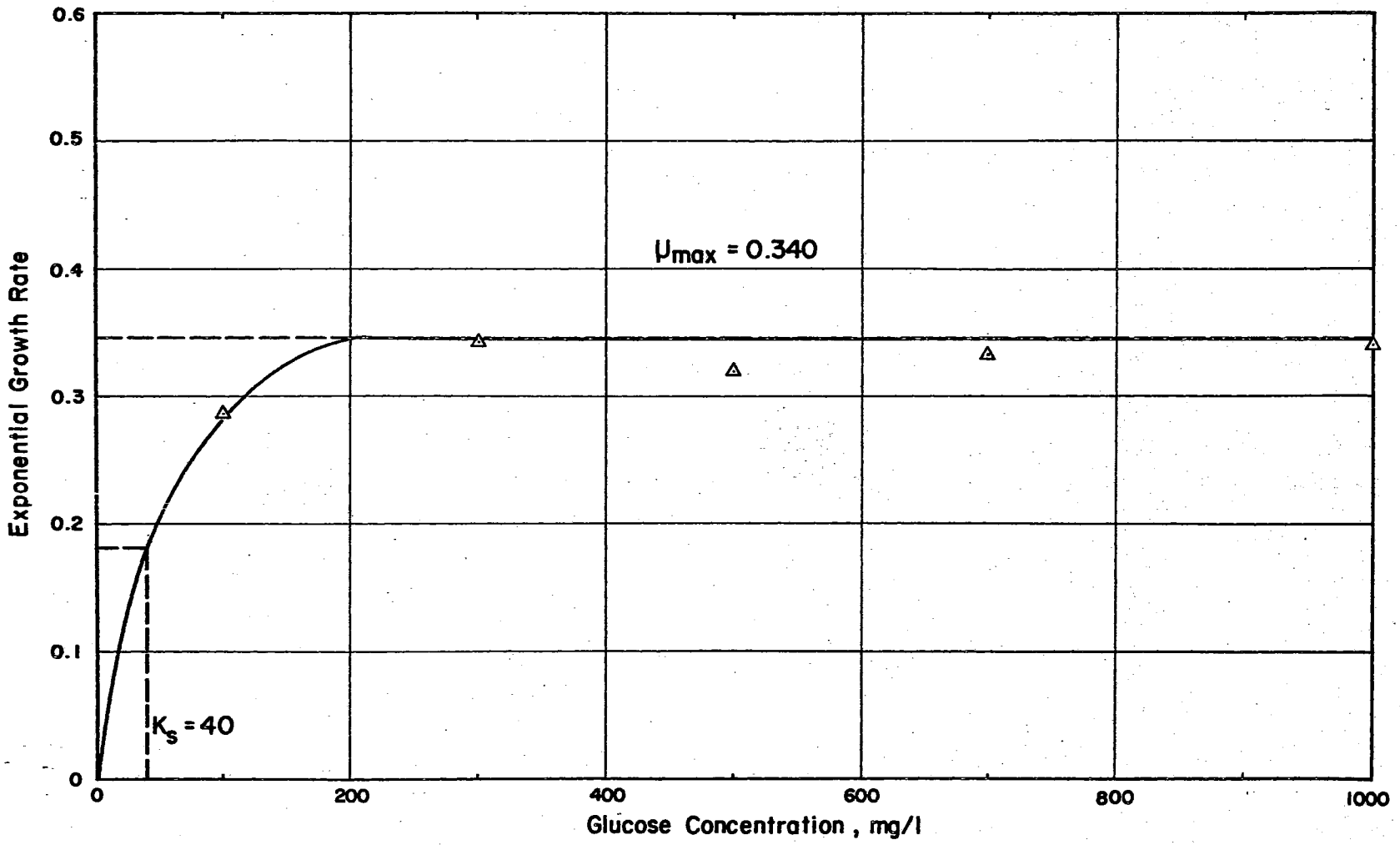

Figure 8. Determination of μ_{max} and K_s for Pseudomonas aeruginosa.

Figures 9, 10, and 11 represent the growth curves and Monod plot for the Yellow organism. This organism exhibited a low μ_{max} and a high K_s. It was the organism which exhibited the highest K_s value of those studied. This organism, however, was very difficult to study as it tended to flocculate at times for no apparent reason and it was deemed unsuitable for continuous flow studies because it would attach itself to a great degree to the walls of the reactor vessel. Regardless of the specific behavior of this organism, it is felt that the kinetic growth rate constant values computed are reasonably accurate because in the experiments shown no flocculation was observed.

Figures 12 and 13 show growth curves for the organism Serratia marcescens. Figure 14 shows the Monod plot with the calculated values of μ_{max} and K_s.

The growth curves for Escherichia intermedia are shown in the arithmetic plot of Figure 15. A plateau in growth can be observed in this figure, particularly at the substrate concentrations of 500 mg/l and above. The semi-log plot in Figure 16 is used to determine the values of the primary growth rate which is plotted vs. substrate concentration for Escherichia intermedia in Figure 17.

Figures 18 and 19 show the growth curves of an experiment performed with Escherichia coli. The kinetic growth rate constants for this organism are shown in Figure 20.

A tabulation of the results obtained in this section is given in Table II.

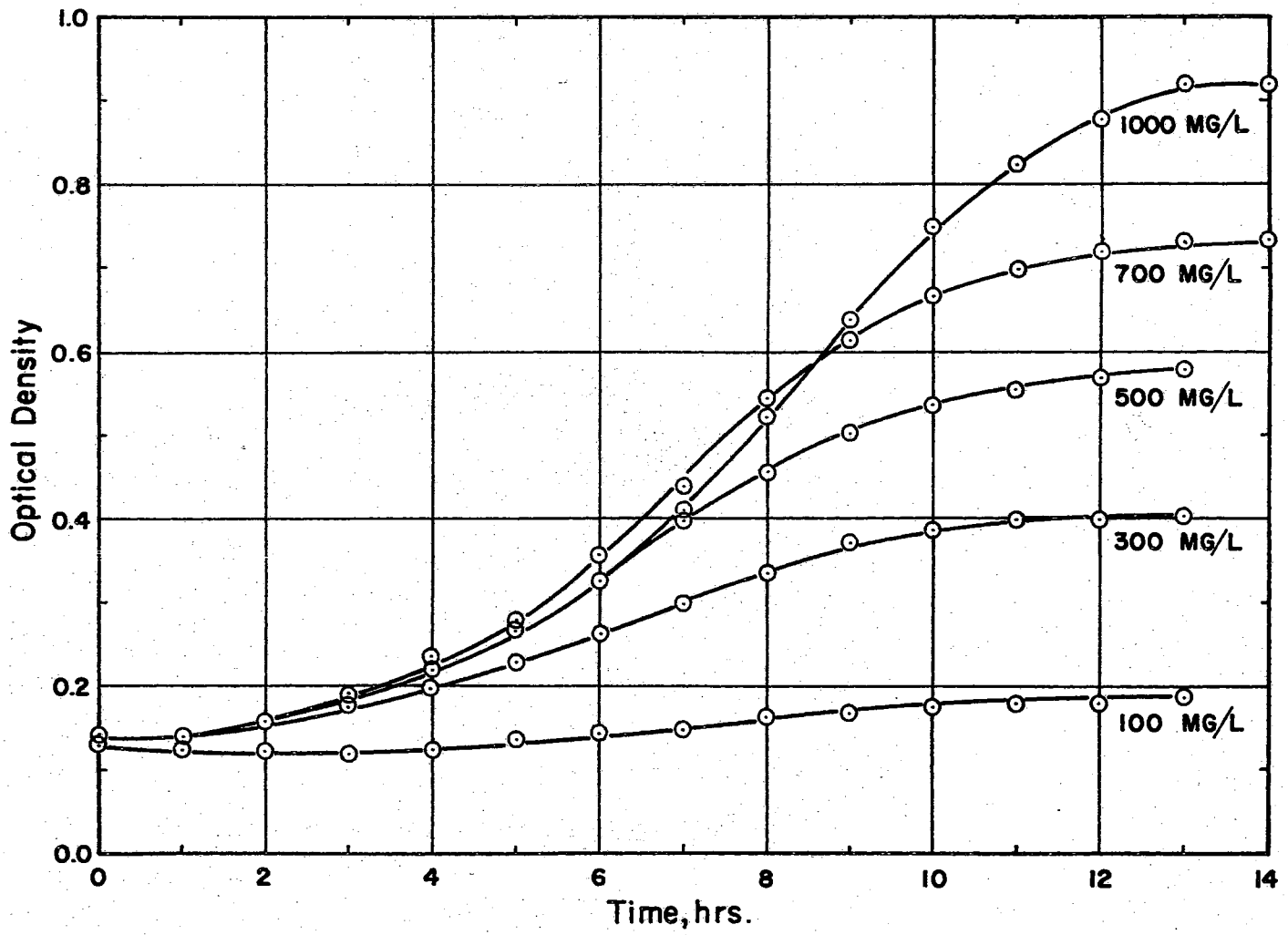

Figure 9. Growth of Yellow organism on various concentrations of glucose.

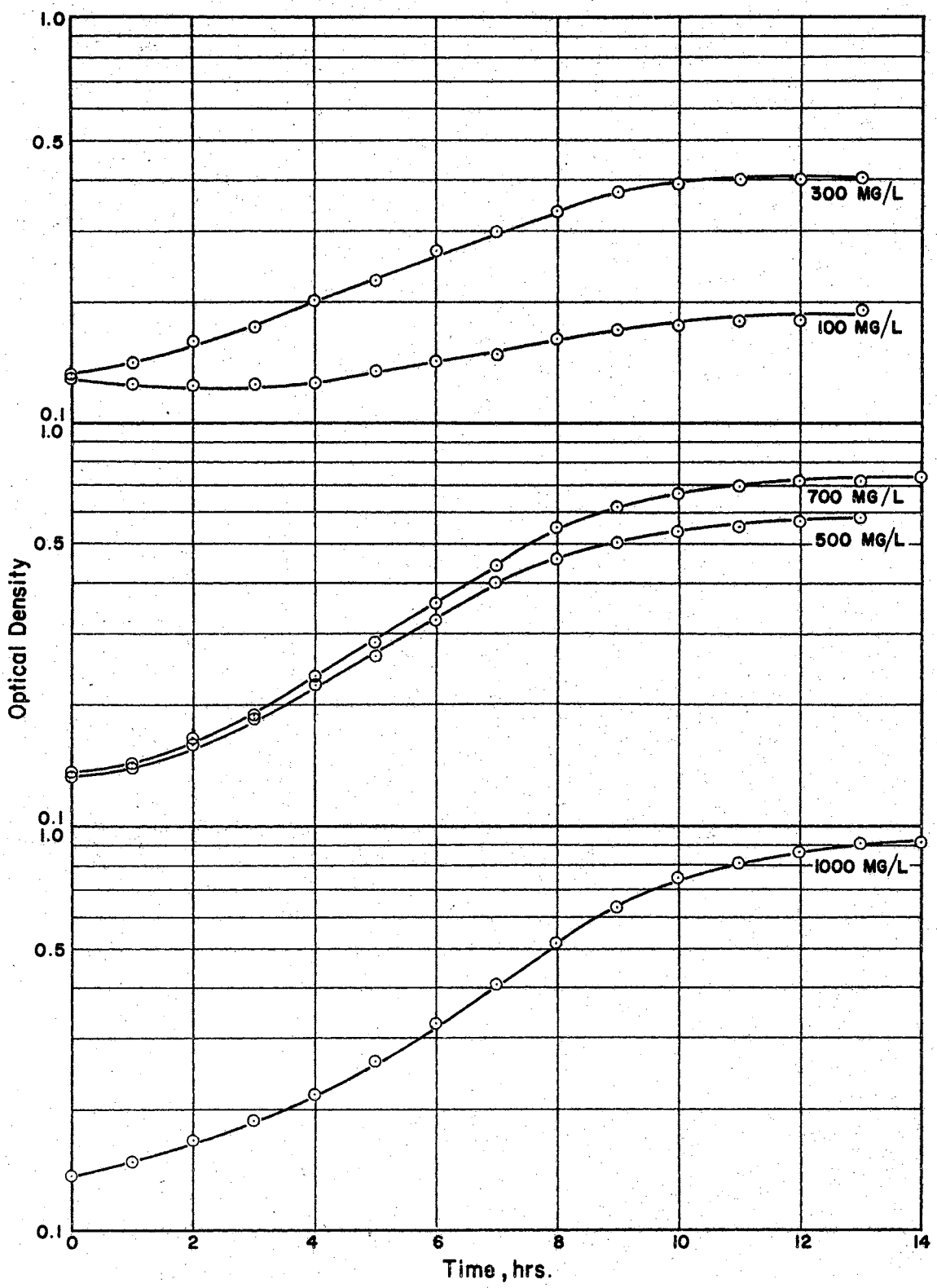

Figure 10. Effects of glucose concentration on growth rate of Yellow organism.

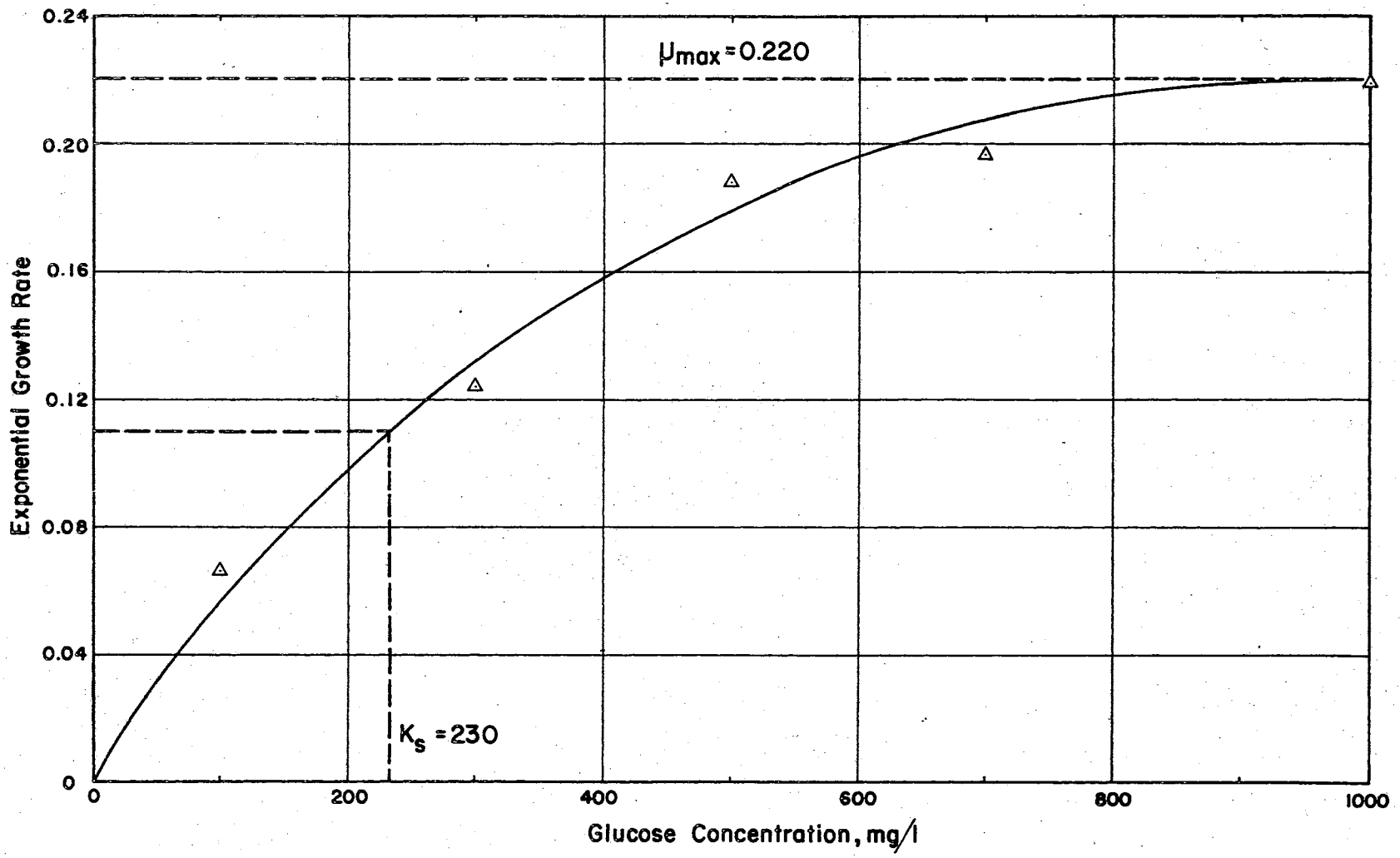

Figure 11. Determination of μ_{max} and K_s for Yellow organism.

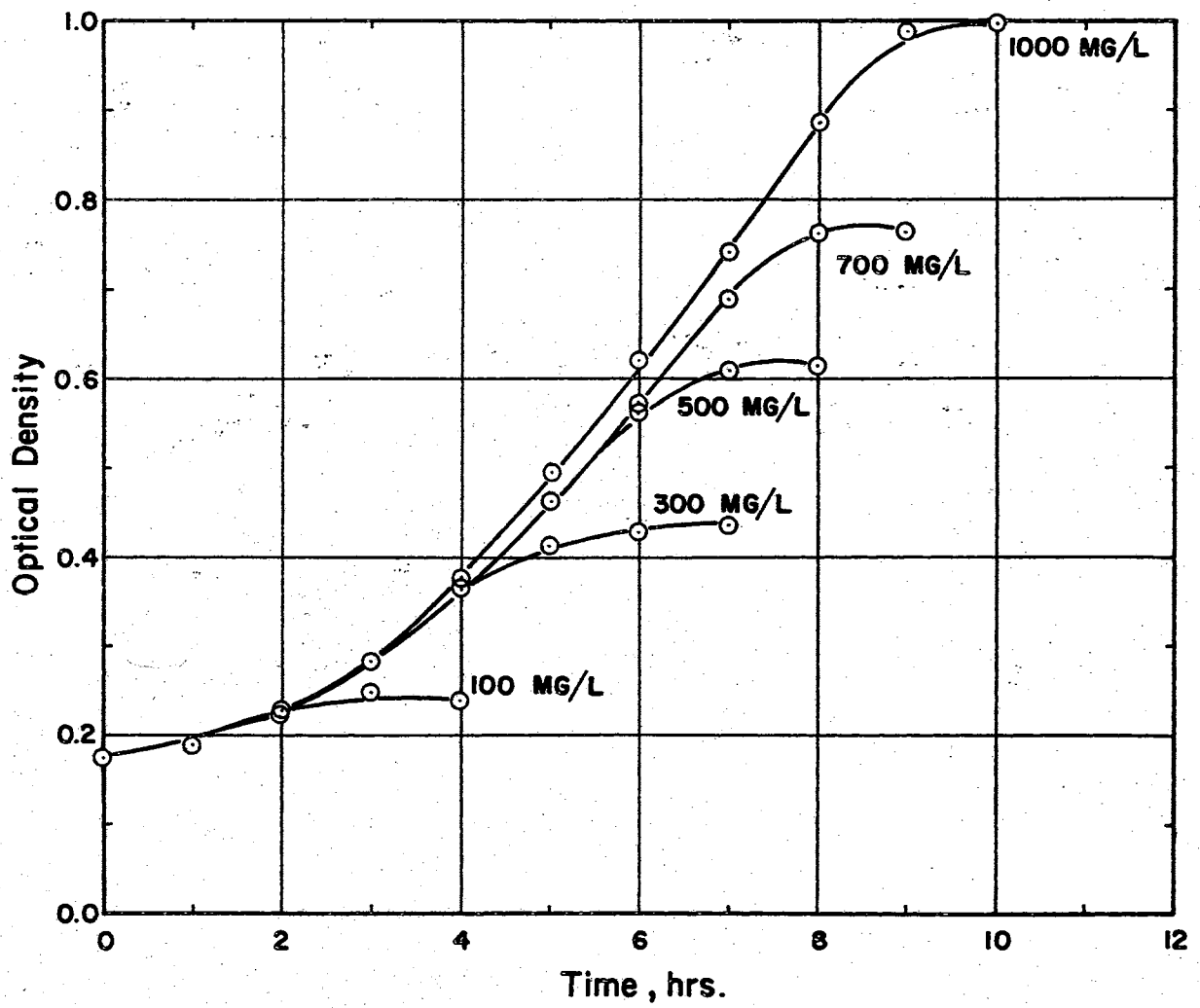

Figure 12. Growth of Serratia marcescens on various concentrations of glucose.

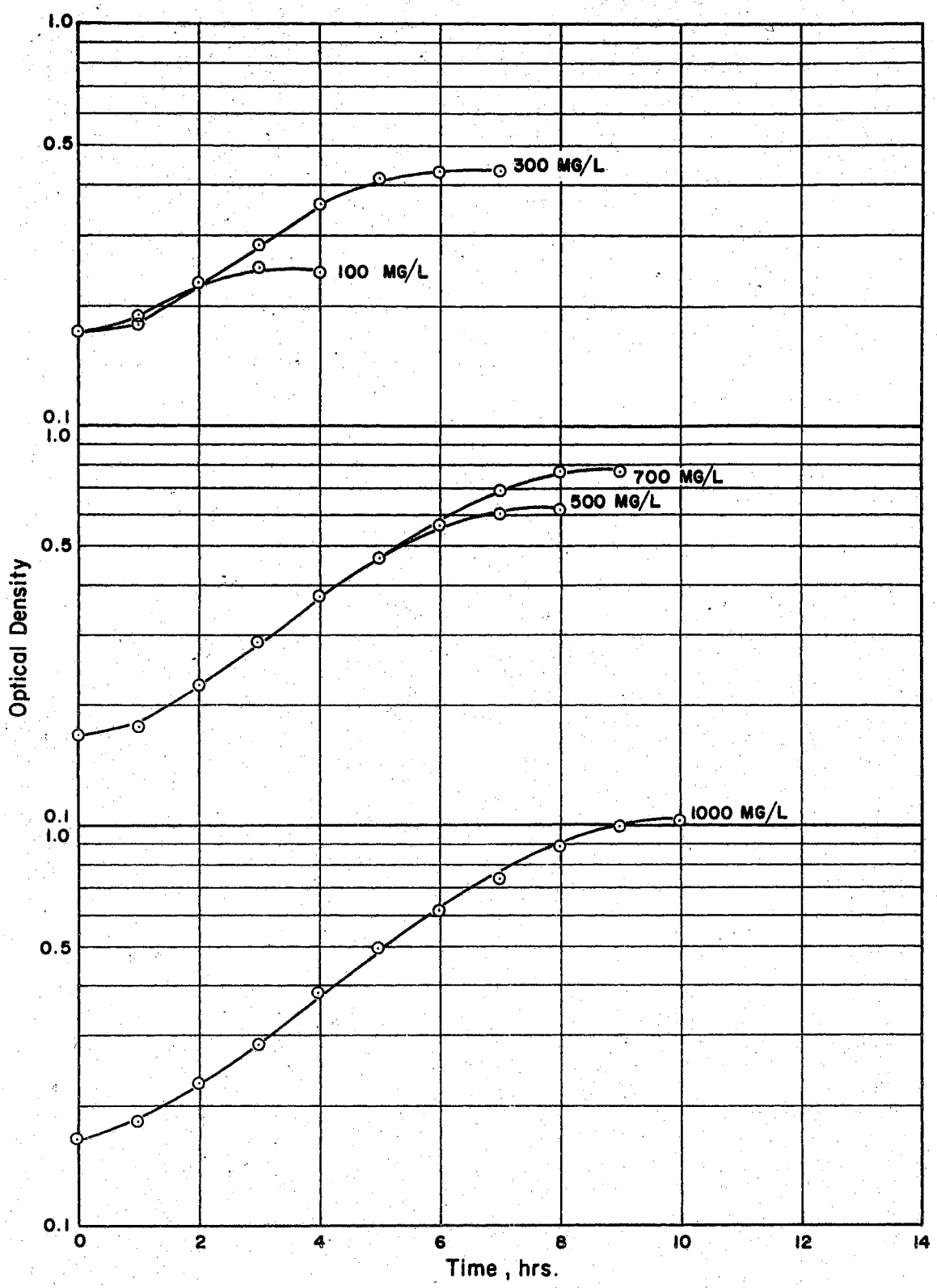

Figure 13. Effects of glucose concentration on growth rate of Serratia marcescens.

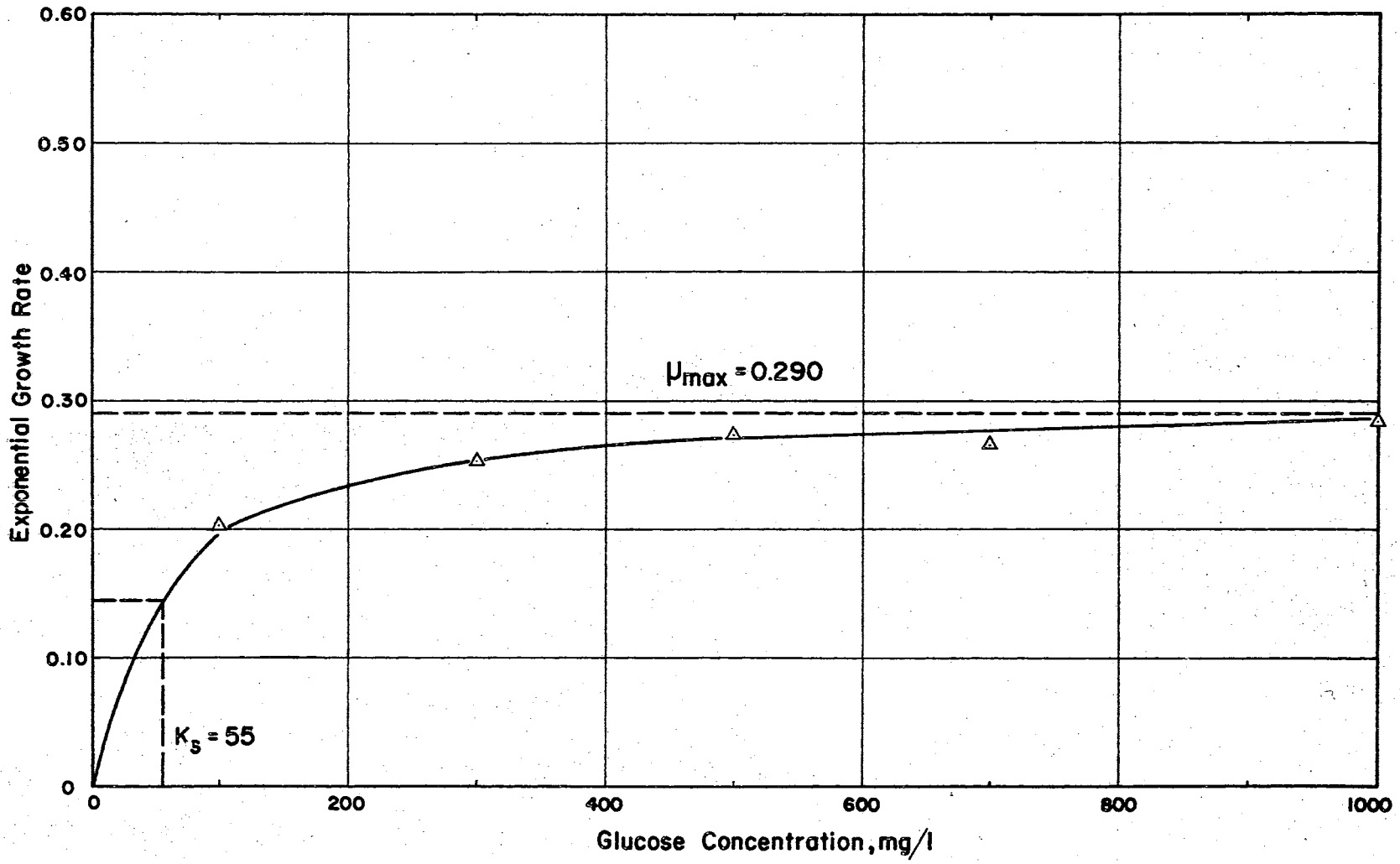

Figure 14. Determination of μ_{max} and K_s for Serratia marcescens.

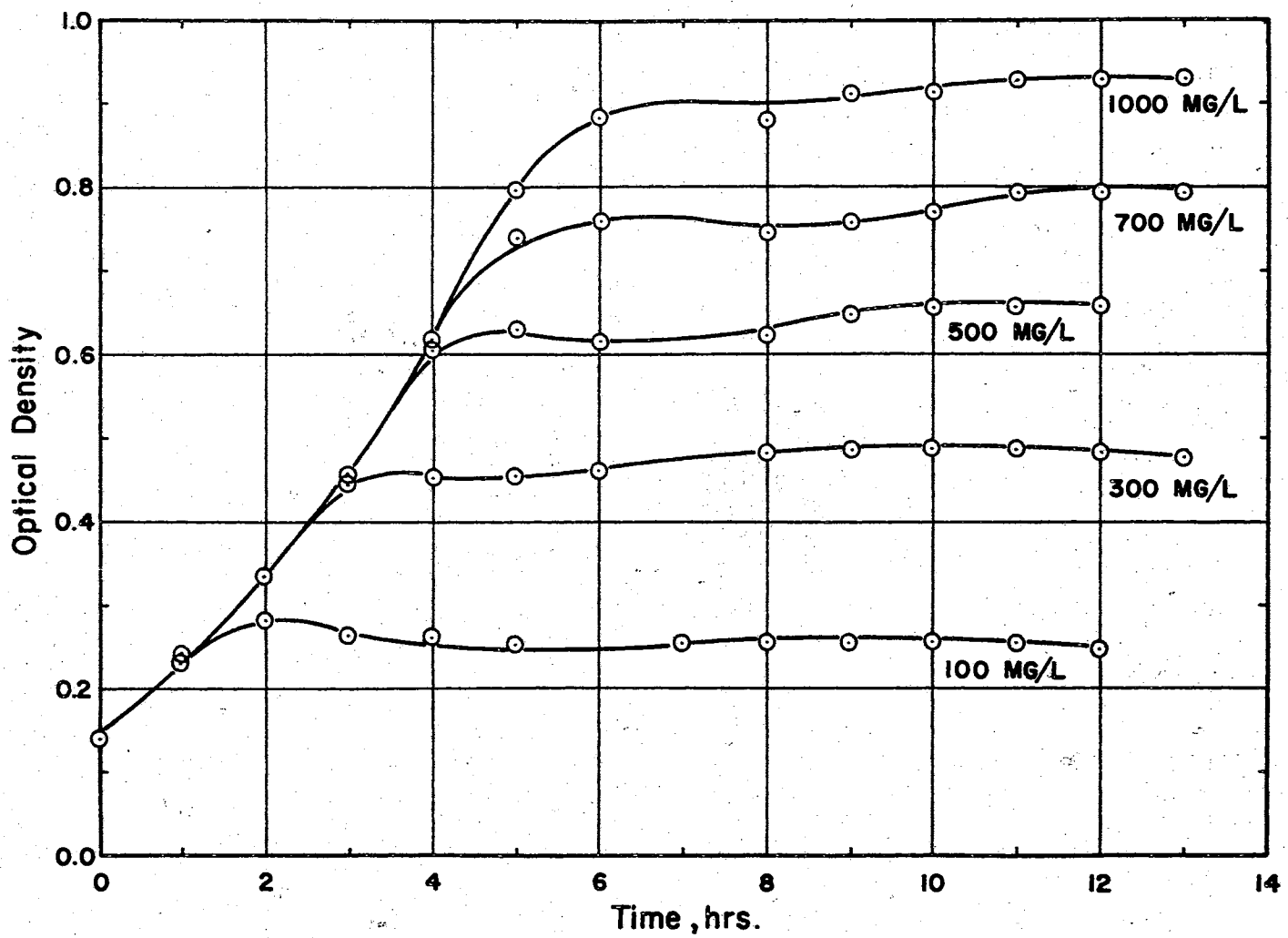

Figure 15. Growth of Escherichia intermedia on various concentrations of glucose.

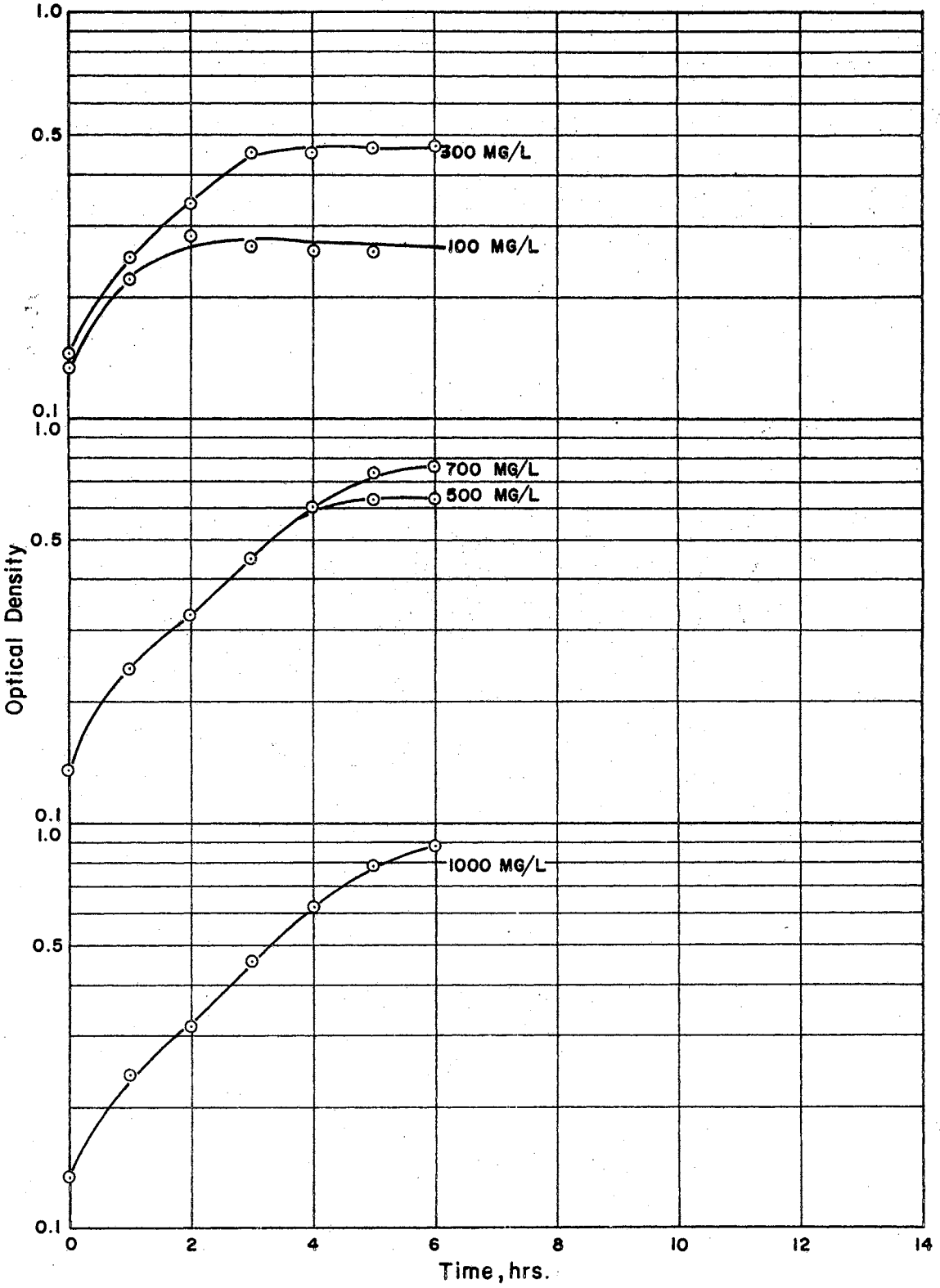

Table 16. Effects of glucose concentration on growth rate of Escherichia intermedia.

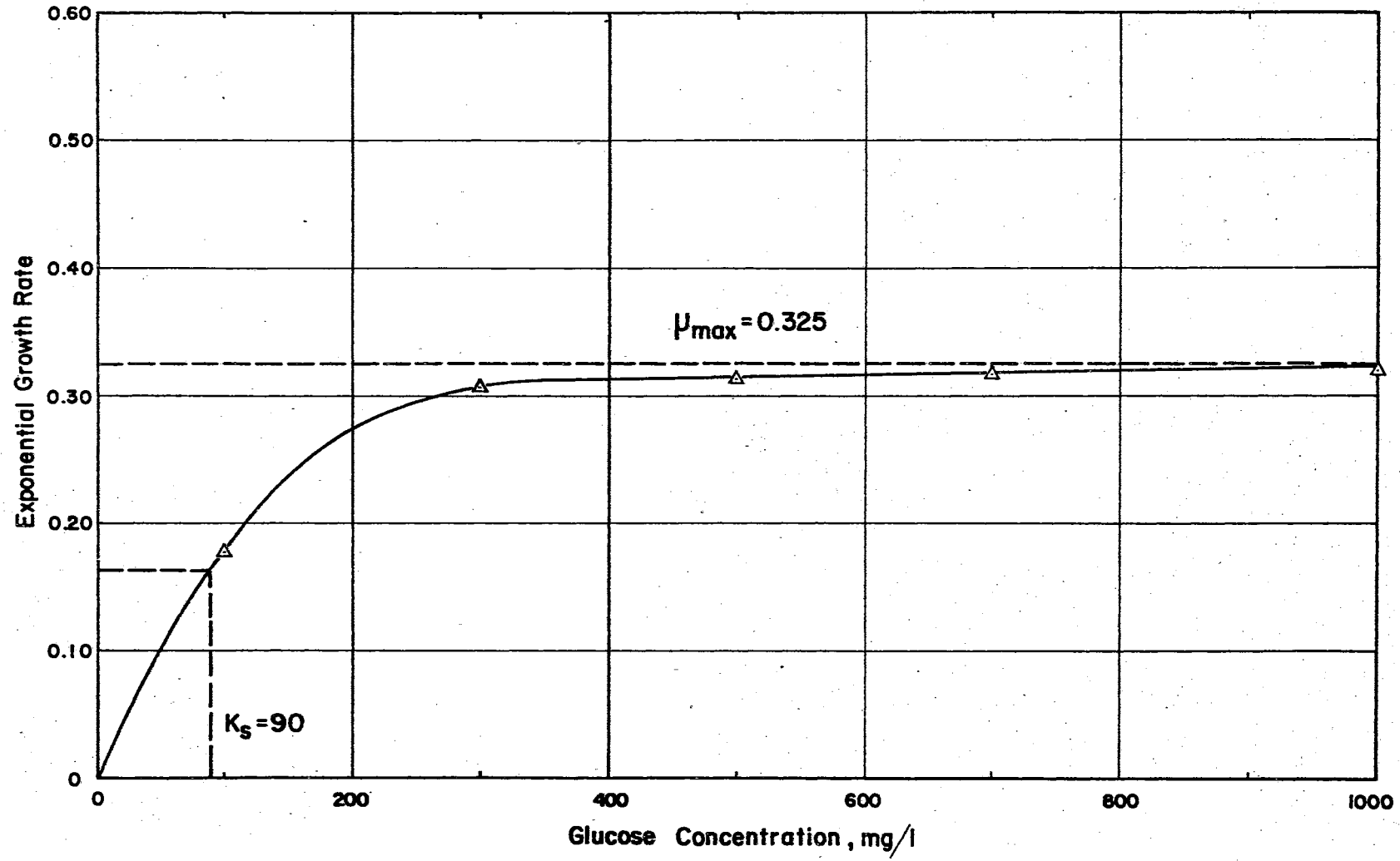

Figure 17. Determination of μ_{max} and K_s for Escherichia intermedia.

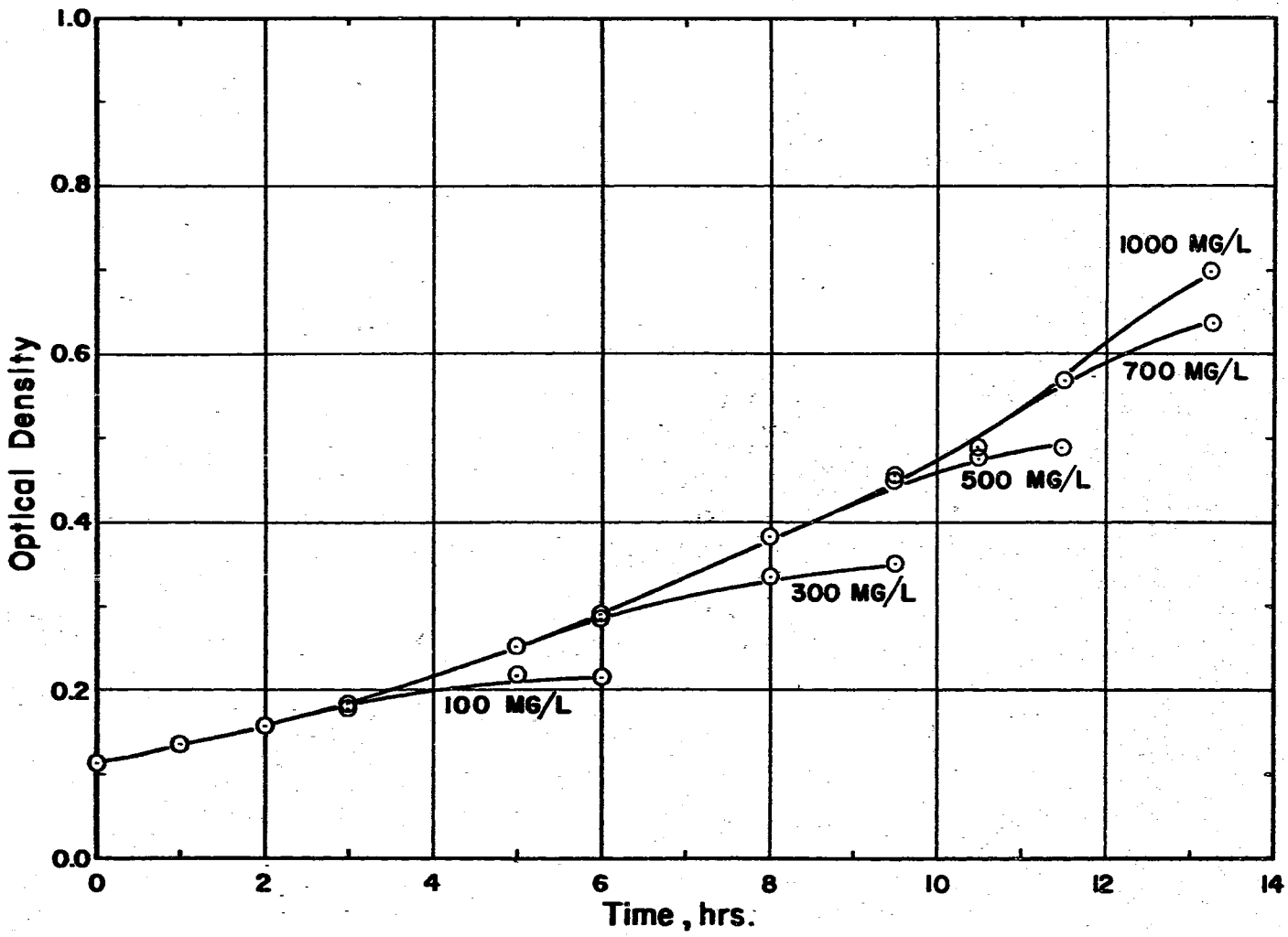

Figure 18. Growth of Escherichia coli K-12 on various concentrations of glucose.

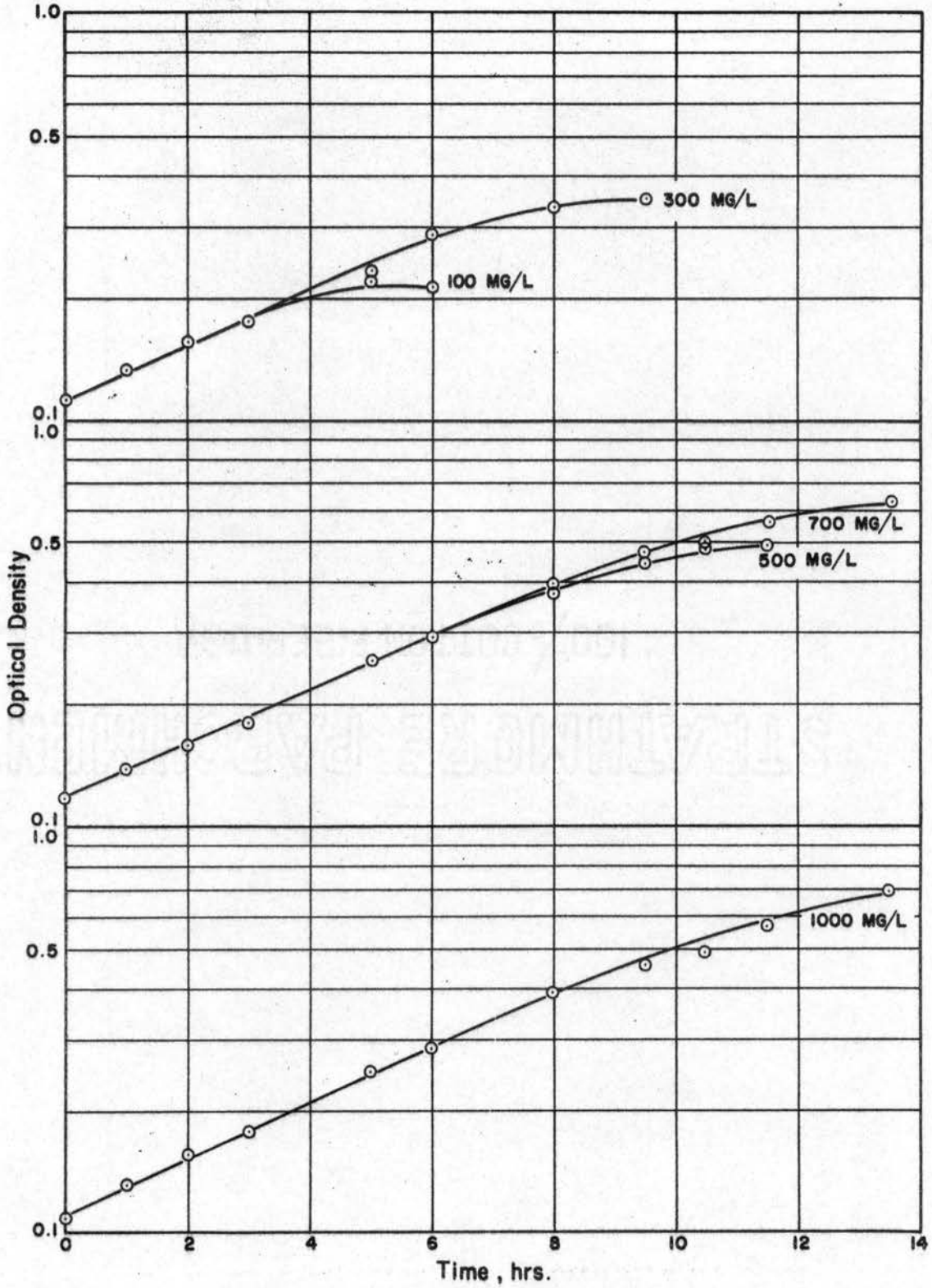

Figure 19. Effects of glucose concentration on growth rate of Escherichia coli K-12.

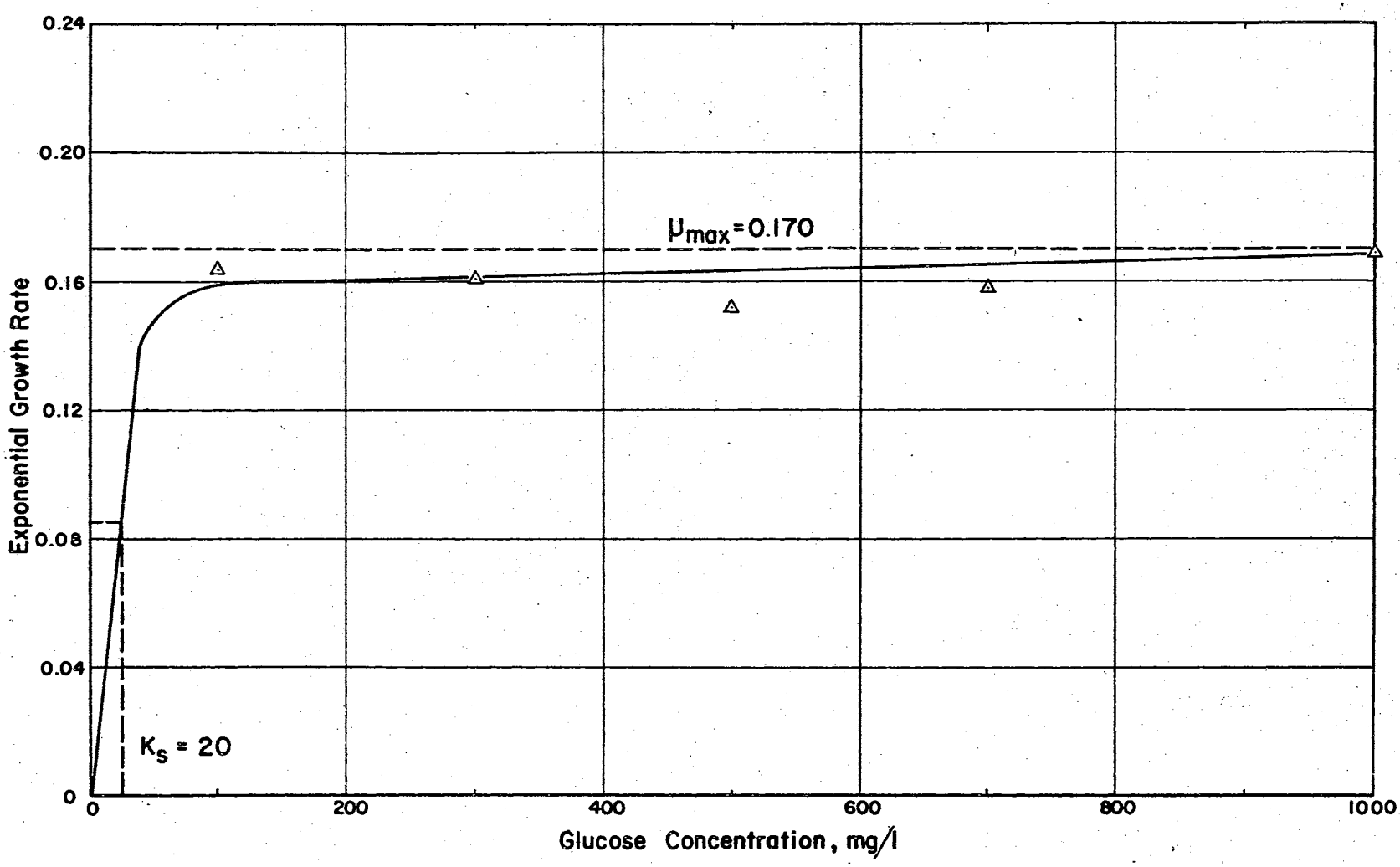

Figure 20. Determination of μ_{max} and K_s for Escherichia coli K-12.

TABLE II

KINETIC CONSTANTS AND CELL YIELD FOR SELECTED ORGANISMS USING GLUCOSE AS SUBSTRATE

Organism	μ_{max}	K_s	Generation Time (hrs)	Cell Yield mg solids/mg glucose	Cell Yield viable cells/mg glusose
Blue organism	0.375	22	0.80	0.697	1.42×10^9
Pseudomonas aeruginosa	0.340	40	0.89	0.482	4.25×10^9
Escherichia intermedia	0.325	90	0.93	0.578	1.49×10^9
Serratia marcescens	0.290	55	1.04	0.447	1.41×10^9
Yellow organism	0.220	230	1.37	0.473	2.65×10^9
Escherichia coli, K-12	0.170	20	1.77	0.424	1.06×10^9

Analysis

The organisms under consideration are listed in Table II in order of decreasing μ_{max} values. This was done because it was felt that μ_{max} would be a principal parameter in attempting to predict predominance patterns in mixture of cultures. The organisms can be classified into three types according to μ_{max} values:

1. Fast-growing organisms: Blue organism, Pseudomonas aeruginosa, and Escherichia intermedia.

2. Medium-fast growing organisms: Serratia marcescens.

3. Slow-growing organisms: Yellow organism, and Escherichia coli.

The relationship between μ_{max} and K_s is not clearly defined by the data collected. There does not appear to be any set pattern of K_s values that can be related to the μ_{max} of each organism. It was noted that the two lowest K_s values correspond to the organisms with the highest and lowest μ_{max} of the strains studied. It was also noted that organisms with a high K_s value would exhibit a significant decrease in their growth rates at low substrate concentration. It could therefore be possible that predominance patterns might be altered at low substrate concentrations if an organism having a high K_s value were present in a mixed culture.

In attempting to compare the yield values with μ_{max}, it was noted that the yield, based on weight of biological

solids produced, was highest in those organisms with high μ_{max}, while it was notably lower in the medium fast and slow-growing organisms. The yield was calculated on the assumption that all of the carbon source had been removed from the medium when the culture showed no further growth. Pseudomonas aeruginosa, however, did not follow such a pattern. Considering the yield as viable cells per unit of substrate, it is seen that there is no apparent pattern in the distribution of yields.

It is interesting to note that the plateau in the growth curve of Figure 15 (Escherichia intermedia) substantiates, using an optical density curve, the existence of the plateau in oxygen uptake observed by Bhatla (107) for the same organism.

2. Kinetic Growth Rate Studies using Sorbitol as Substrate

The organisms that were studied using sorbitol as substrates were: Blue organism, Serratia marcescens, and Escherichia intermedia. As Pseudomonas aeruginosa and Yellow organism did not grow on sorbitol, they are not considered in this section.

Figure 21 shows the growth curves for the Blue organism plotted on semi-log paper. The exponential growth rate is plotted vs. substrate concentration in Figure 22 for this organism.

Figures 23 and 24 show the growth rate curves and Monod plot for Serratia marcescens.

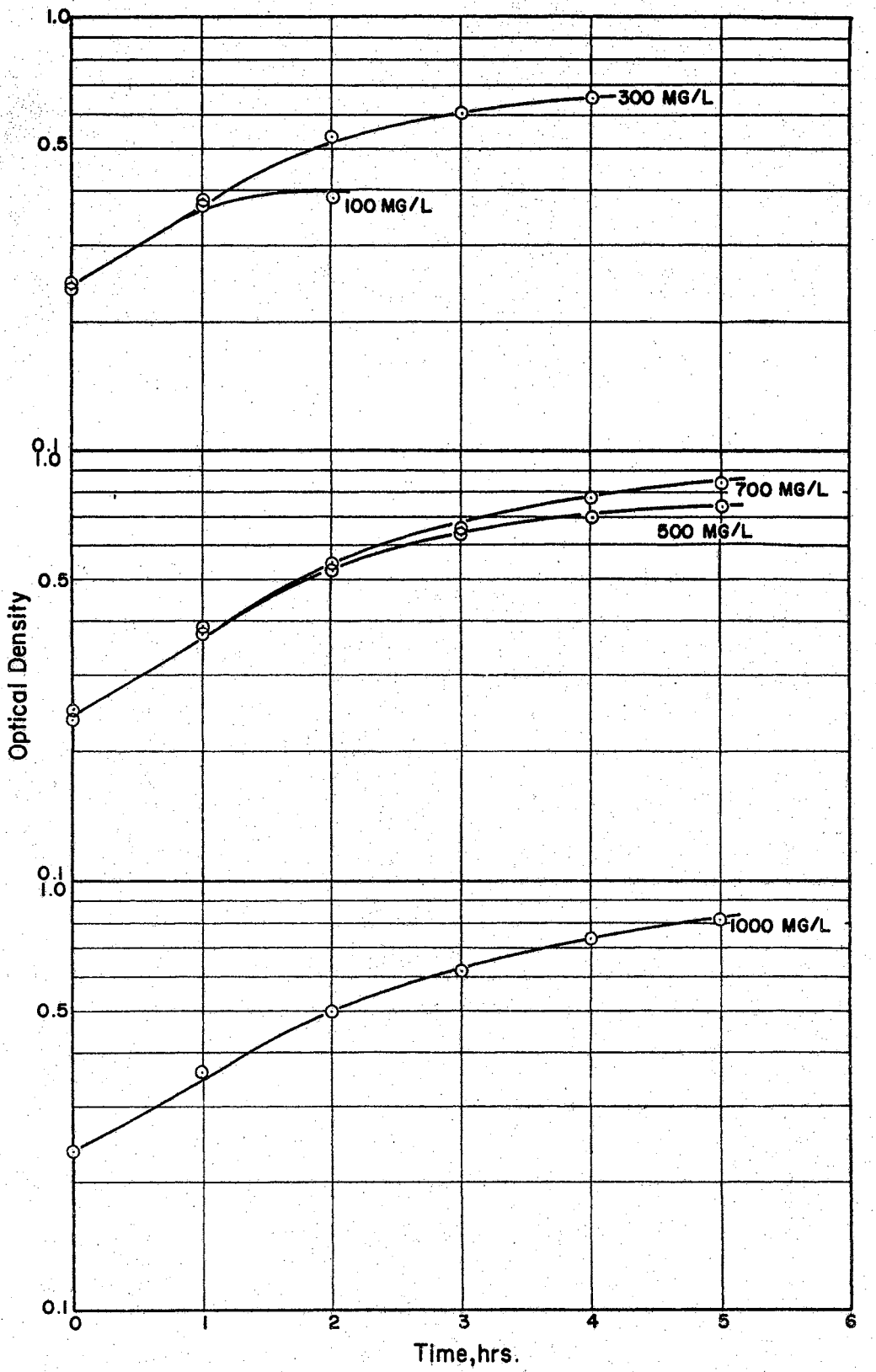

Figure 21. Effects of sorbitol concentration on growth rate of Blue organism.

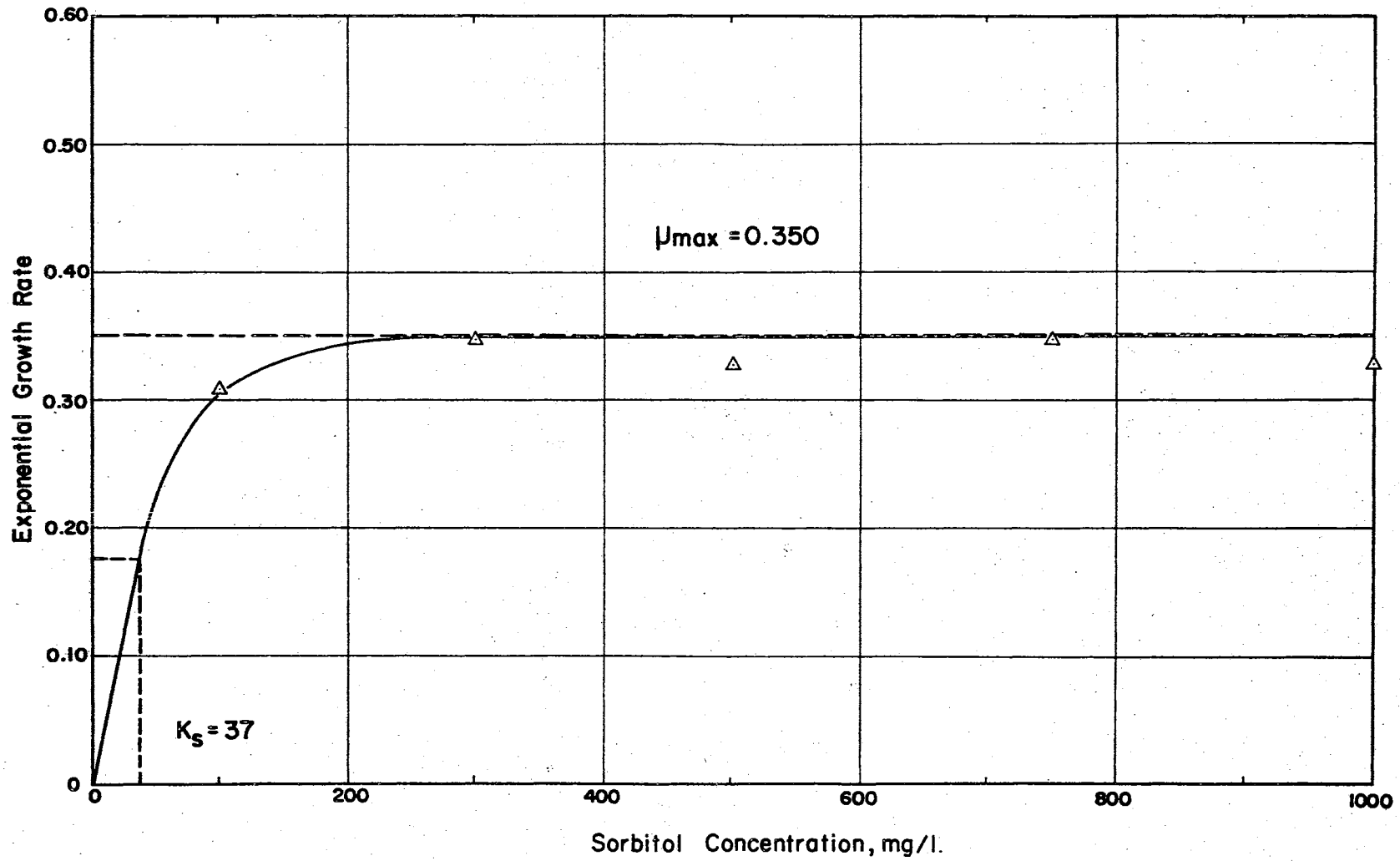

Figure 22. Determination of μ_{max} and K_s for Blue organism using sorbitol as substrate.

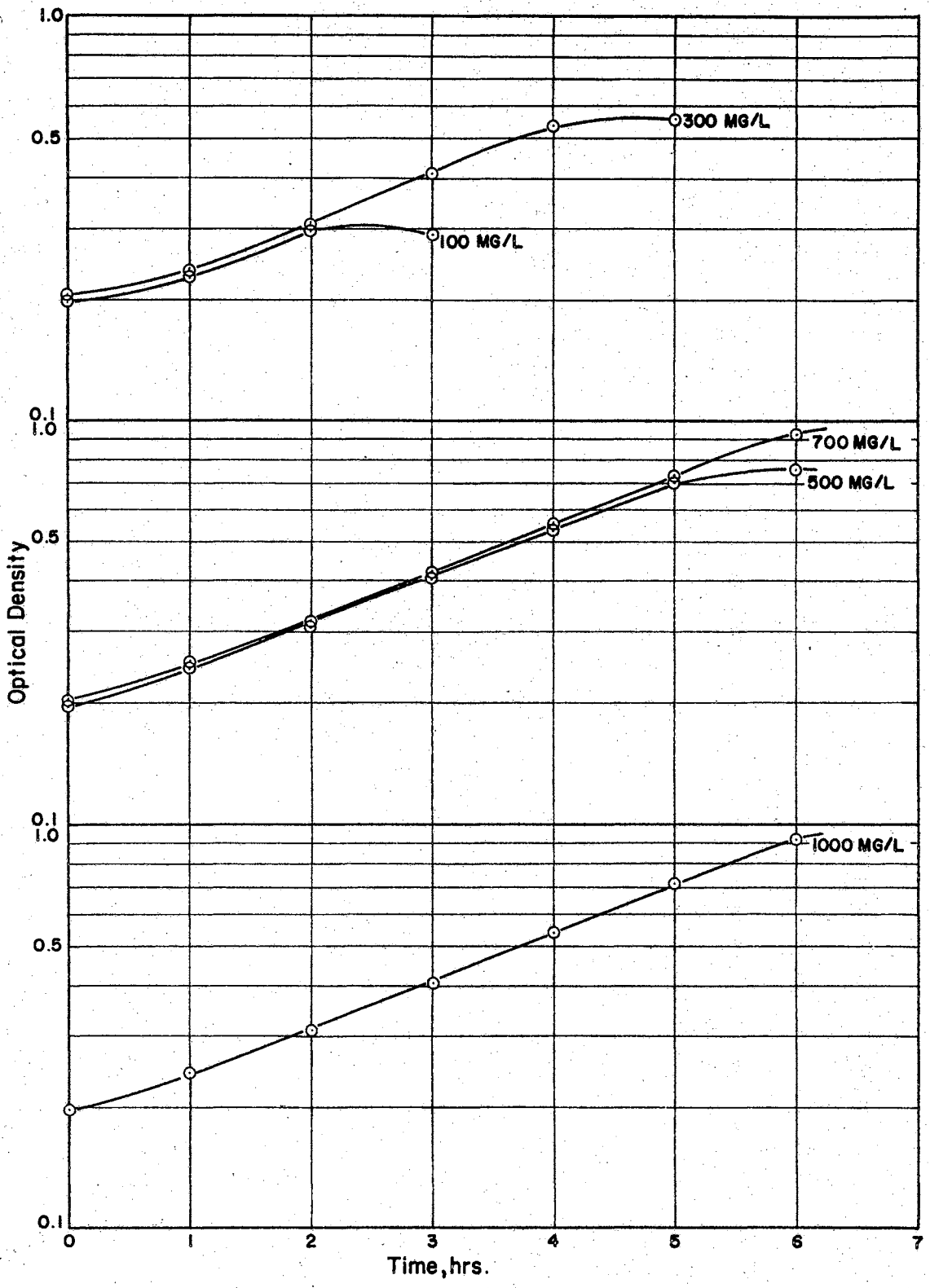

Figure 23. Effects of sorbitol concentration on growth rate of Serratia marcescens.

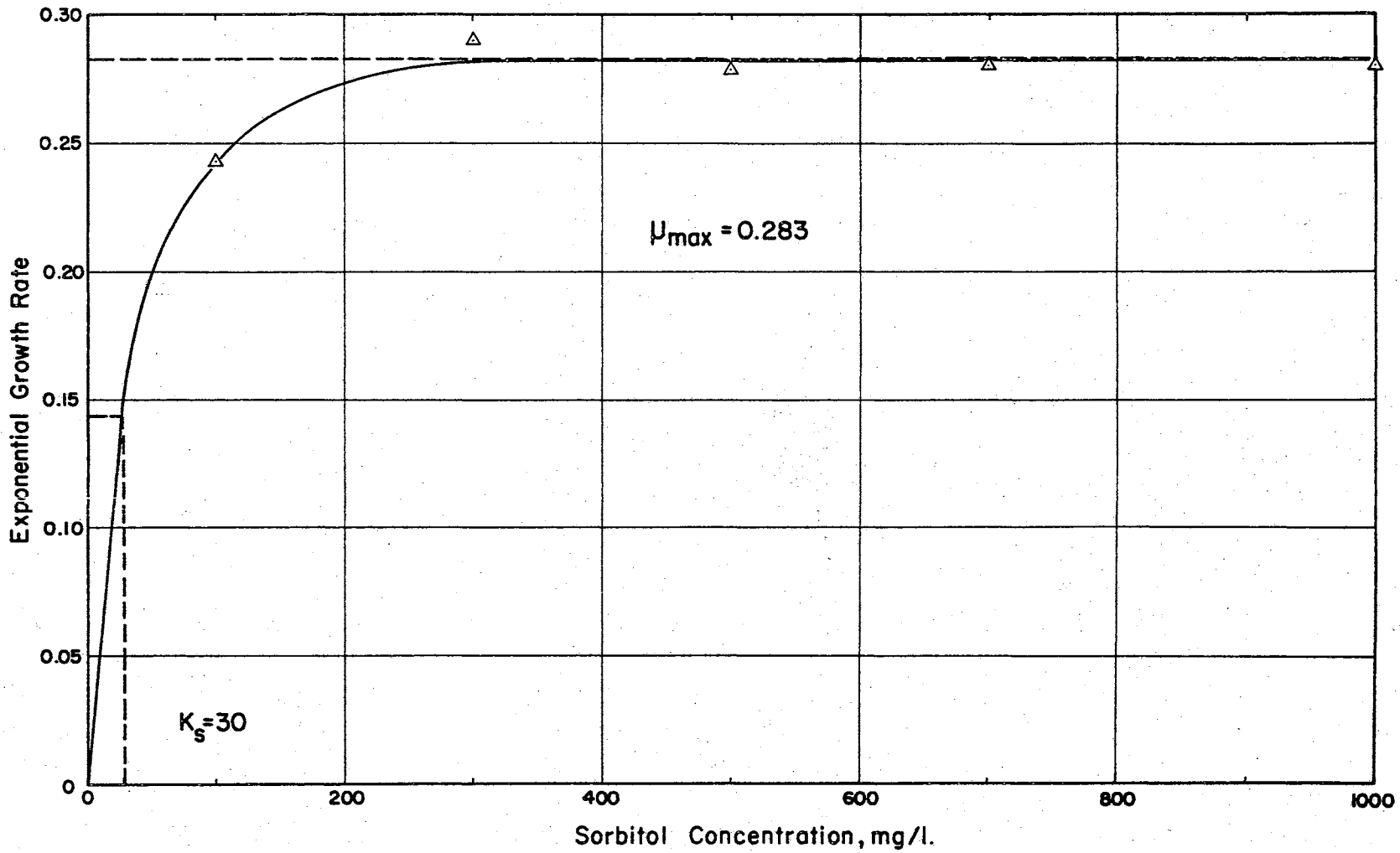

Figure 24. Determination of μ_{max} and K_s for Serratia marcescens using sorbitol as substrate.

Figures 25 and 26 show the growth rate curves and Monod plot for Escherichia intermedia. Although the arithmetic growth rate plot for this organism is not shown, it was noted that there was no evidence for the plateau in growth that was observed when glucose was used as substrate.

A tabulation of the results obtained in this section is given in Table III, where the organisms are listed in order of decreasing μ_{max} values.

Analysis

An examination of the kinetic constants and yield values contained in Table III indicates that the values obtained using sorbitol as substrate were similar to those obtained with glucose (Table II). If the three organisms considered were classified based on their μ_{max} values on sorbitol, Blue organism and Escherichia intermedia would be classified as fast growers, and Serratia marcescens would be a medium fast-growing organism. In relation to the K_s values obtained, it appears that K_s for Escherichia intermedia on sorbitol is somewhat lower than that observed with glucose.

Of interest is the observation that no growth plateau was registered with Escherichia intermedia. Such a plateau is represented, in glucose metabolism, by the period of time necessary for the organism to acclimate itself to the metabolic intermediates accumulated. The absence of a plateau for the same organism in sorbitol may indicate that no intermediates were produced and accumulated during

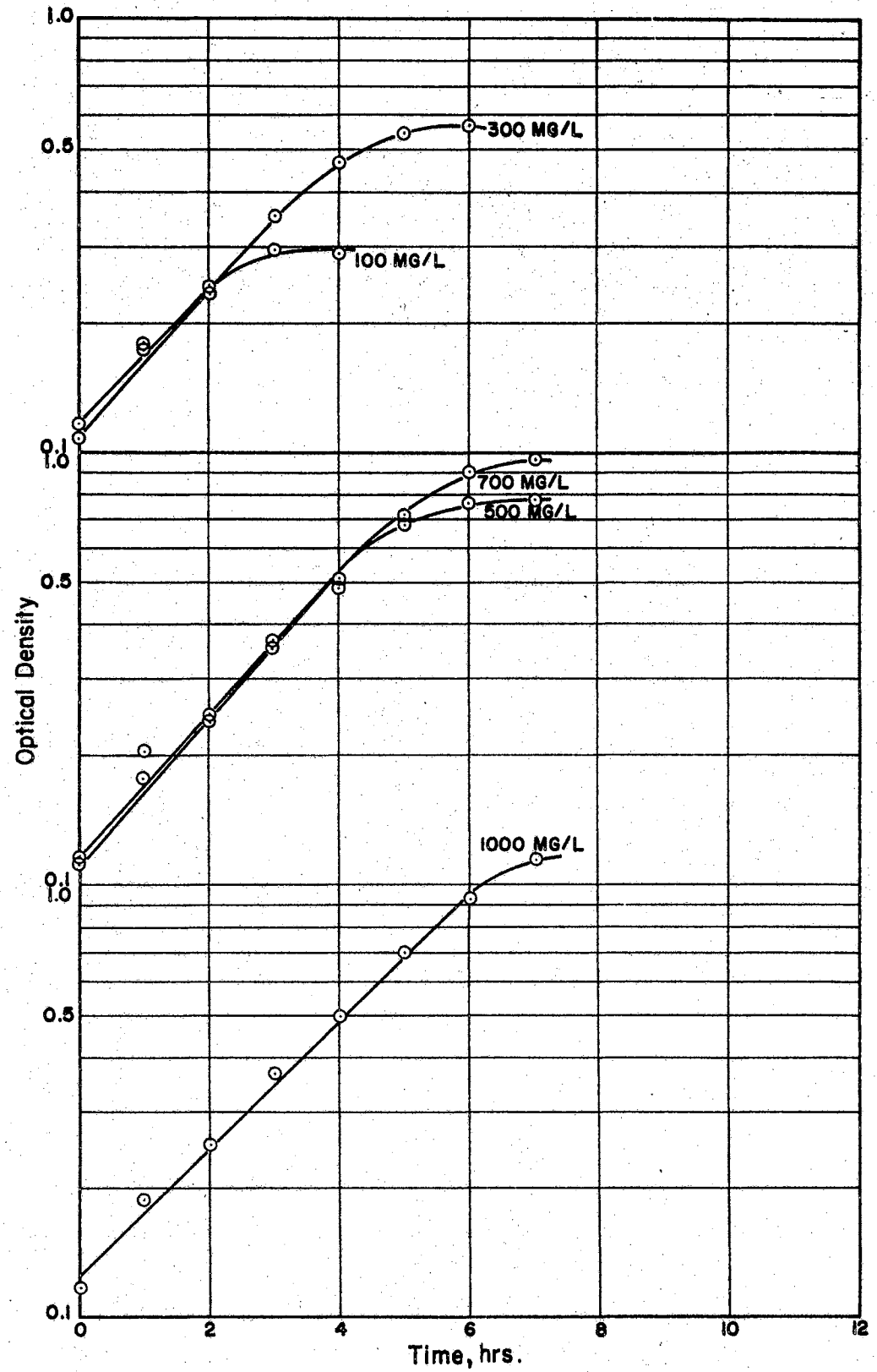

Figure 25. Effects of sorbitol concentration on growth of Escherichia intermedia.

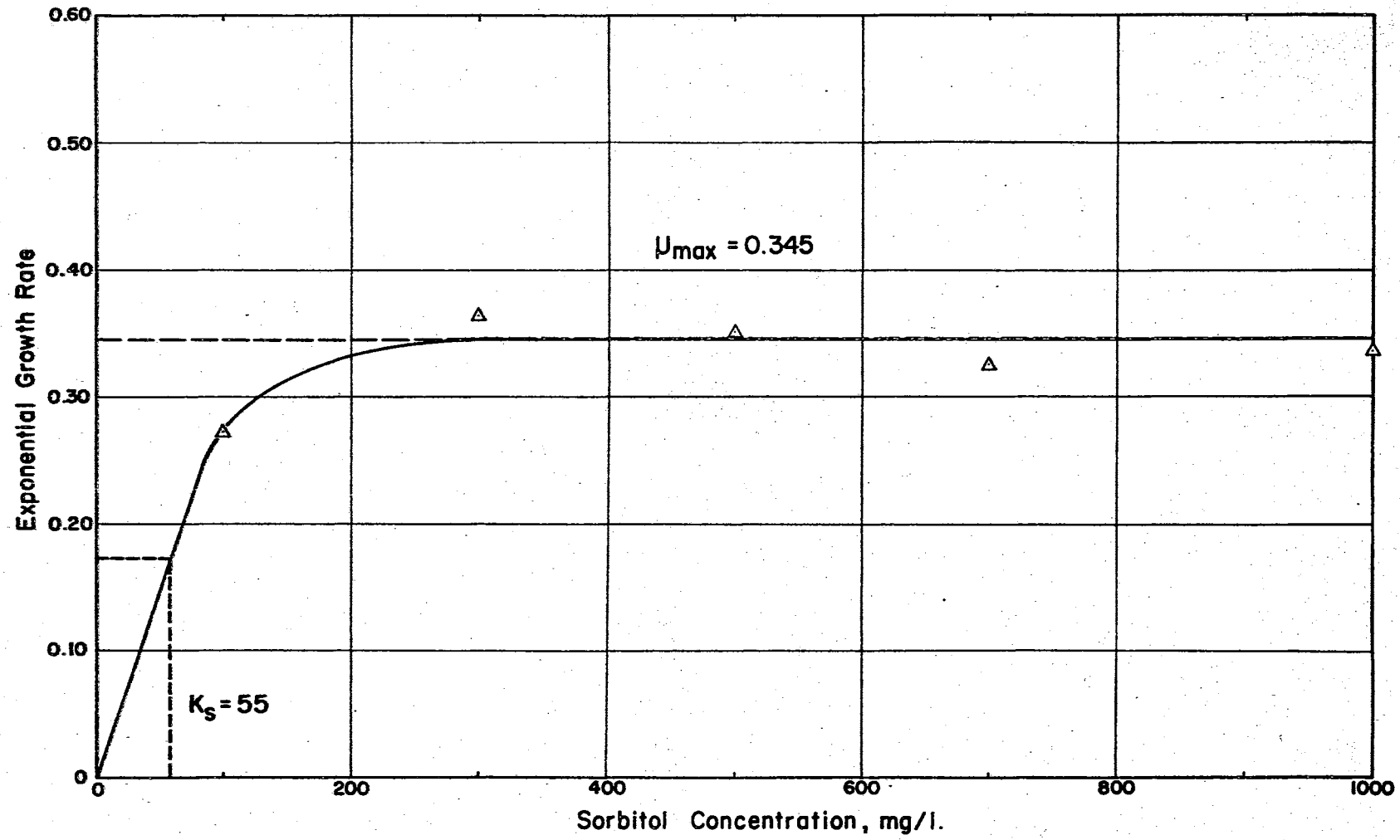

Figure 26. Determination of μ_{max} and K_s for Escherichia intermedia using sorbitol as substrate.

metabolism of that carbohydrate.

TABLE III

KINETIC CONSTANTS AND CELL YIELD FOR SELECTED ORGANISMS USING SORBITOL AS SUBSTRATE

Organism	μ_{max}	K_s	Generation Time (hrs)	Cell Yield viable cells/mg sorbitol
Blue organism	0.350	37	0.86	1.26×10^9
Escherichia intermedia	0.345	55	0.89	1.16×10^9
Serratia marcescens	0.283	30	1.06	1.58×10^9

3. Use of Reciprocal Plots for the Determination of μ_{max} and K_s

Reciprocal plots similar to those recommended by Lineweaver and Burk (100) are often used for the calculation of μ_{max} and K_s. In order to compare the values obtained using both procedures, plots of $1/\mu$ against 1/S were made for the computation of the kinetic constants of the organisms studied. Figure 27 illustrates the use of the Lineweaver-Burk plot for one of the organisms (Serratia marcescens), with glucose as substrate. The results are presented in Table IV.

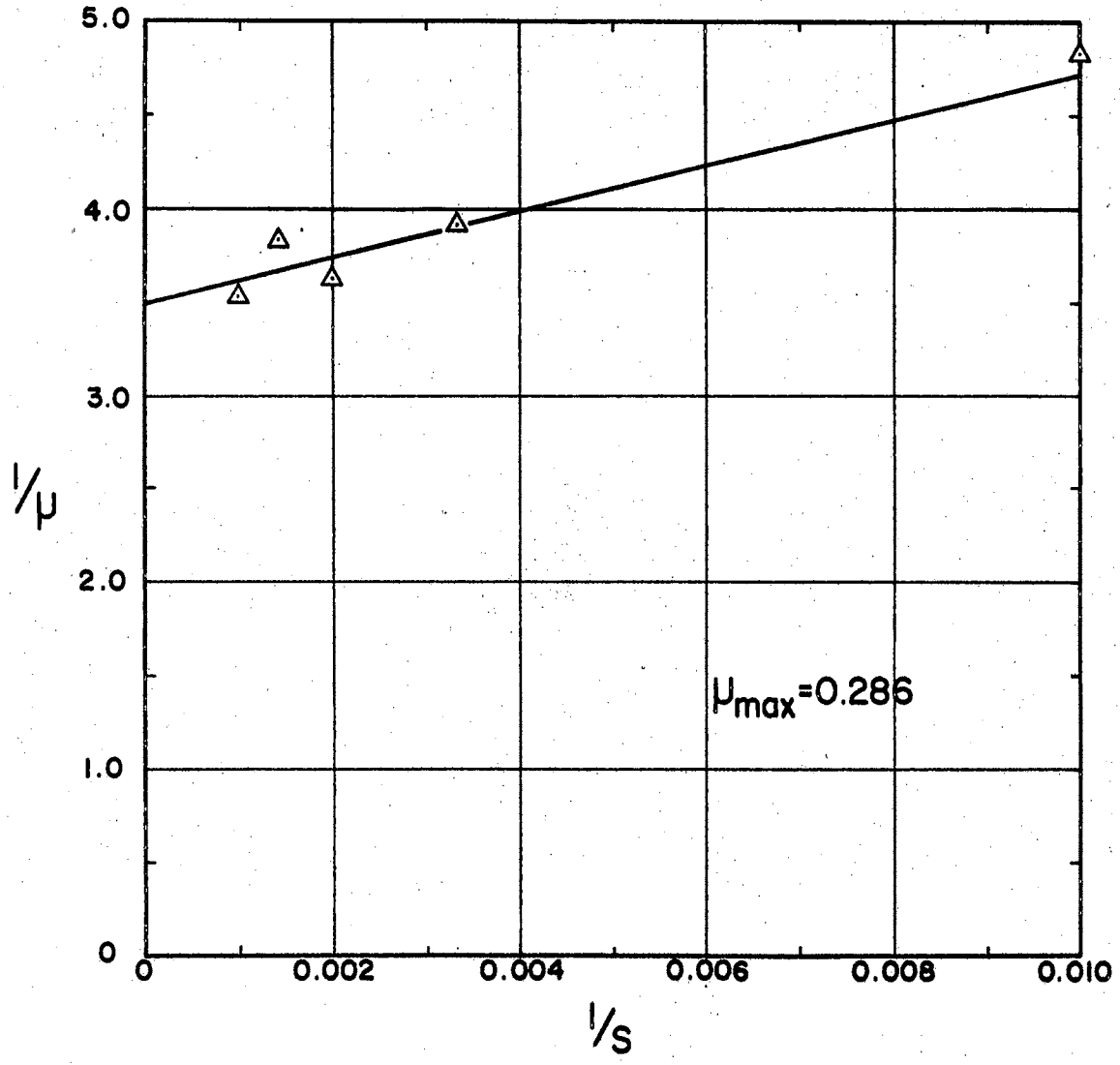

Figure 27. Determination of kinetic constants by the reciprocal plot for Serratia marcescens.

TABLE IV

KINETIC CONSTANTS FOR SELECTED ORGANISMS CALCULATED BY THE RECIPROCAL PLOT METHOD

Organism	Glucose		Sorbitol	
	μ_{max}	K_s	μ_{max}	K_s
Blue organism	0.387	16	0.362	17
Pseudomonas aeruginosa	0.331	13	-	-
Escherichia intermedia	0.342	69	0.345	28
Serratia marcescens	0.286	35	0.290	20
Yellow organism	0.285	322	-	-
Escherichia coli	0.167	13	-	-

Analysis

Comparison of the results presented in Tables II, III, and IV show that in most cases the values of μ_{max} obtained by either method were, with the exception of the Yellow organism, similar in magnitude. In this case, the determination of μ_{max} by the Lineweaver-Burk plot would place the organism among the medium-fast growers rather than with the slow-growing group.

In the comparison of K_s values obtained from the two types of plots, it was seen that in some instances, e.g., the Blue organism and Escherichia coli on glucose, and Serratia marcescens on sorbitol, the differences were very small. Only in the case of the Yellow organism were the K_s differences large. With the Yellow organism, it would seem that if the experiments had included substrate concentrations beyond 1000 mg/l, K_s values, as well as μ_{max} values, may have been more comparable. However, it was not possible to use higher substrate concentrations because the organism tended to flocculate at higher substrate concentrations.

The use of reciprocal plots for the determination of K_s and μ_{max} has been studied extensively by Ramanathan (108). He found that such plots gave better results than those obtained with regular Monod plots, particularly insofar as K_s determinations were concerned. In the experiments herein reported, it is believed that reciprocal plots provided a better approximation of the growth constants. In subsequent analyses in which comparative growth rates were considered, the values obtained from the Lineweaver-Burk plot were used.

In the classification of organisms by their μ_{max} values as fast growers, slow growers, and medium-fast growers, it should be pointed out that these terms imply only a very rough approximation of their growth characteristics. This classification, however, was found to be

useful in the presentation and comparison of results of mixed culture experiments.

4. Acclimation Experiments

These experiments were undertaken to determine if the various species of bacteria being studied would grow on a variety of carbohydrates. Two to four ml of a bacterial suspension grown on glucose were transferred to flasks containing 1000 mg/l of various carbohydrates and incubated on the shaker at 25°C. Several transfers were attempted if the organism did not grow on the first seeding of a specific carbohydrate in order to make certain of the results. The cultures that did not grow were allowed to incubate for at least one week before being discarded. The carbohydrates used were sorbitol, glycerol, galactose, sorbose, sucrose, ribose, mannitol, and fructose. The results of these experiments are shown in Table V.

Analysis

It is noted that the fast growers, Blue organism and Escherichia intermedia, grew on all of the substrates which were examined. On the other hand, the medium-fast grower, Serratia marcescens, did not grow on sorbose, while Yellow organism metabolized only a few of the carbohydrates. Those organisms with a high μ_{max} on glucose grew well on all substrates used, while those with a lower μ_{max} did not grow with some of the carbohydrates. Pseudomonas aeruginosa, however, is the exception among the microorganisms studied, because it did not grow on any of the substrates

tested with the exception of glycerol, fructose, and mannitol. Because of its high μ_{max} on glucose and because of its lack of ability to grow on many of the carbohydrates tested, Pseudomonas aeruginosa was used extensively in mixed cultures experiments to determine the behavior of the culture when incubated with other organisms in the presence of a substrate that it could not assimilate.

TABLE V

ABILITY OF BACTERIAL CULTURES TO UTILIZE VARIOUS SUBSTRATES

Organism	Sorbitol	Glycerol	Galactose	Sorbose	Sucrose	Ribose	Mannitol	Fructose
Blue organism	+	+	+	+	+	+	+	+
Serratia marcescens	+	+	+	-	+	+	+	+
Escherichia intermedia	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	-	+	-	-	-	-	+	+
Escherichia coli, K-12	+	+						
Yellow organism	+	+	-	-	-	-	-	+

5. Growth Rate Comparison Experiments

These experiments were carried out by acclimating an organism to two different substrates and transferring the acclimated seeds to shaker flasks containing each substrate and a mixture of the substrates. Six curves were thus obtained. The semi-log plots used to calculate specific growth rates (μ) are not presented; however, the results of such calculations are shown on the arithmetic plots. In all cases, substrate concentrations of 700 mg/l were used with the flasks containing both substrates prepared in such a manner that 350 mg/l of each substrate were present.

Results from the first of these experiments are presented in Figure 28. The organism used was Escherichia intermedia, and the two substrates were glucose and sorbitol. It can be seen that the μ values of the glucose-acclimated set and the sorbitol-acclimated set were very similar. In both cases, i.e., with sorbitol-acclimated and glucose-acclimated seed, there was some indication of a secondary growth cycle in the flasks containing glucose. No plateau was observed in the growth curves with sorbitol or with glucose-sorbitol substrate for sorbitol-acclimated seed. However, a distinct break in the growth pattern was observed in the glucose-sorbitol system with a glucose-acclimated seed. It is interesting to note that two types of growth plateau may be operative in the glucose-acclimated system; e.g., one in the glucose control, possibly caused by secondary uptake of intermediates

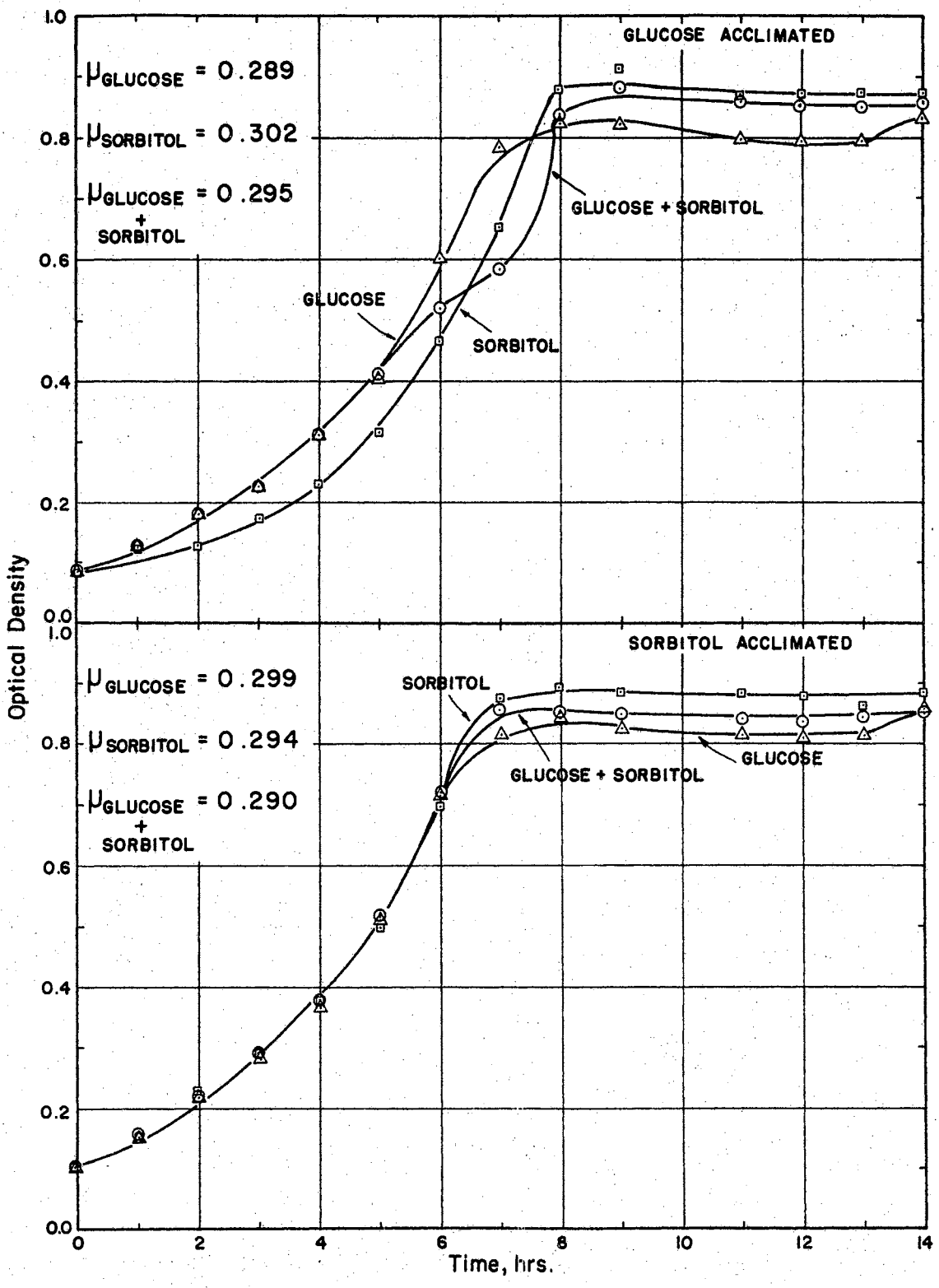

Figure 28. Exponential growth rate comparison of Escherichia intermedia on glucose and sorbitol.

produced during metabolism of glucose and a second in the glucose-sorbitol curve probably caused by sequential substrate removal of each substrate originally placed in the system.

The results of an experiment using Blue organism grown on glucose and sorbitol are presented in Figure 29. For both systems the growth rates on single substrates (glucose or sorbitol) are approximately the same. The growth rate in the multicomponent system for sorbitol-acclimated cells is somewhat lower than its glucose-acclimated counterpart. A slight plateau in the growth curve can be observed in the two-substrate, glucose-acclimated system; no plateau was noted in any of the remaining curves.

Figure 30 shows the results obtained in an experiment with Serratia marcescens grown on glucose and sorbitol. The exponential growth rates were higher for the sorbitol-acclimated set using glucose as substrate and lower on the glucose substrate. No plateau was observed in any of these curves, and a distinct lag period was observed on sorbitol with glucose-acclimated cells.

In Figure 31, the results for a system consisting of Blue organism on glucose and glycerol are presented. The μ values on glycerol for both sets of experiments are similar. The exponential growth rates for the glucose-acclimated cells in the glucose and glucose-glycerol systems are higher than those of the glycerol-acclimated set. Both multicomponent substrate curves exhibited a plateau regardless of

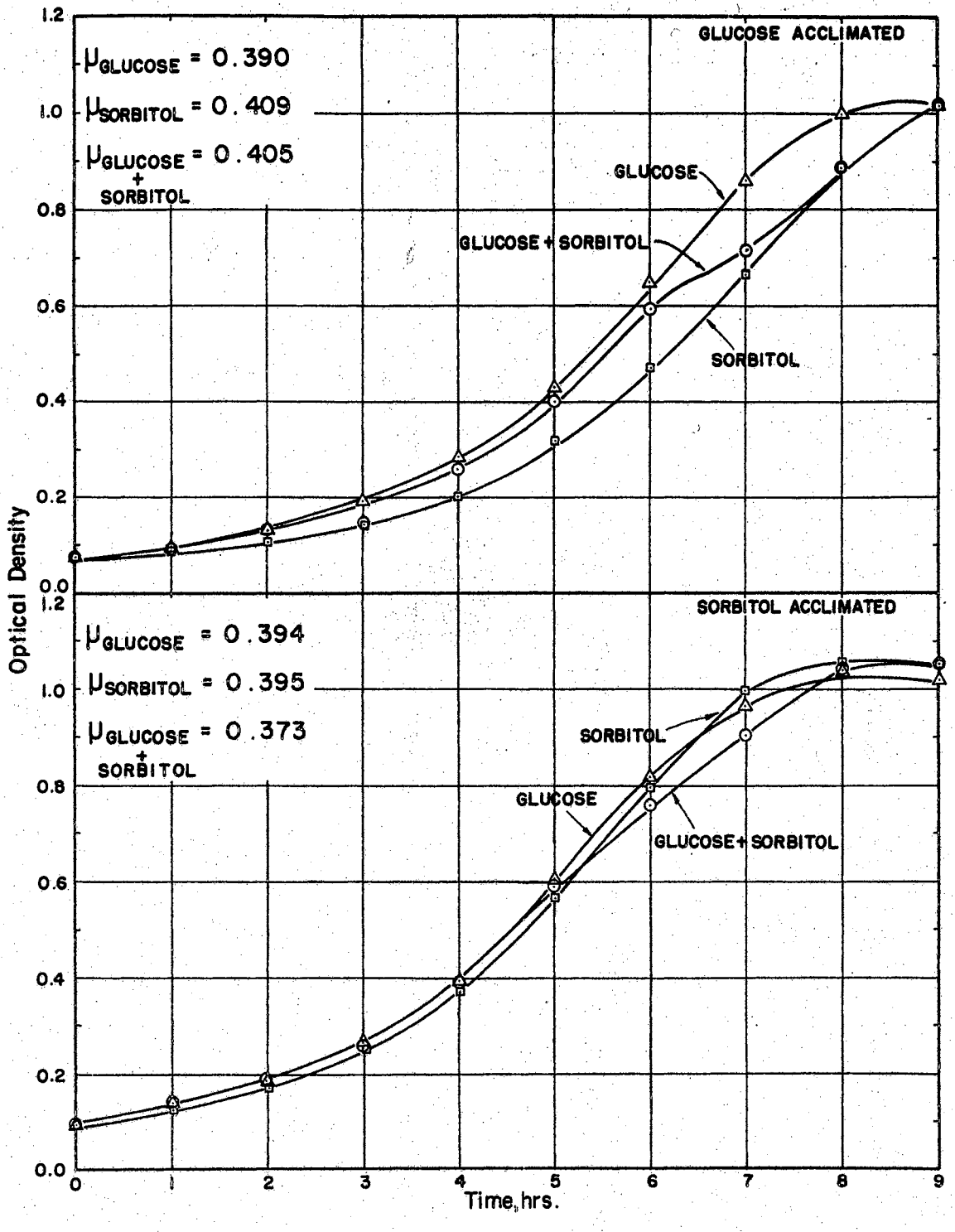

Figure 29. Exponential growth rate comparison of Blue organism on glucose and sorbitol.

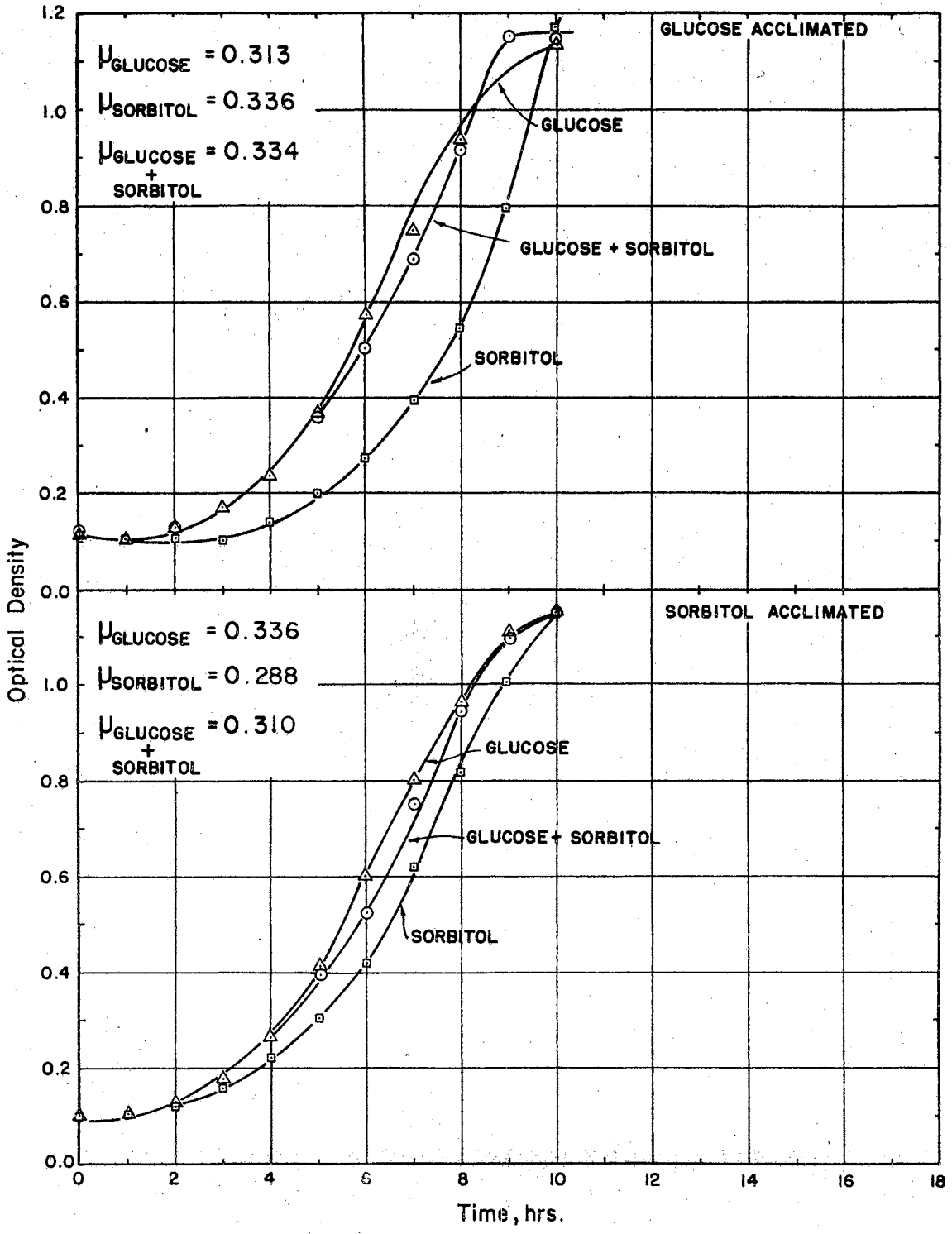

Figure 30. Exponential growth rate comparison of Serratia marcescens on glucose and sorbitol.

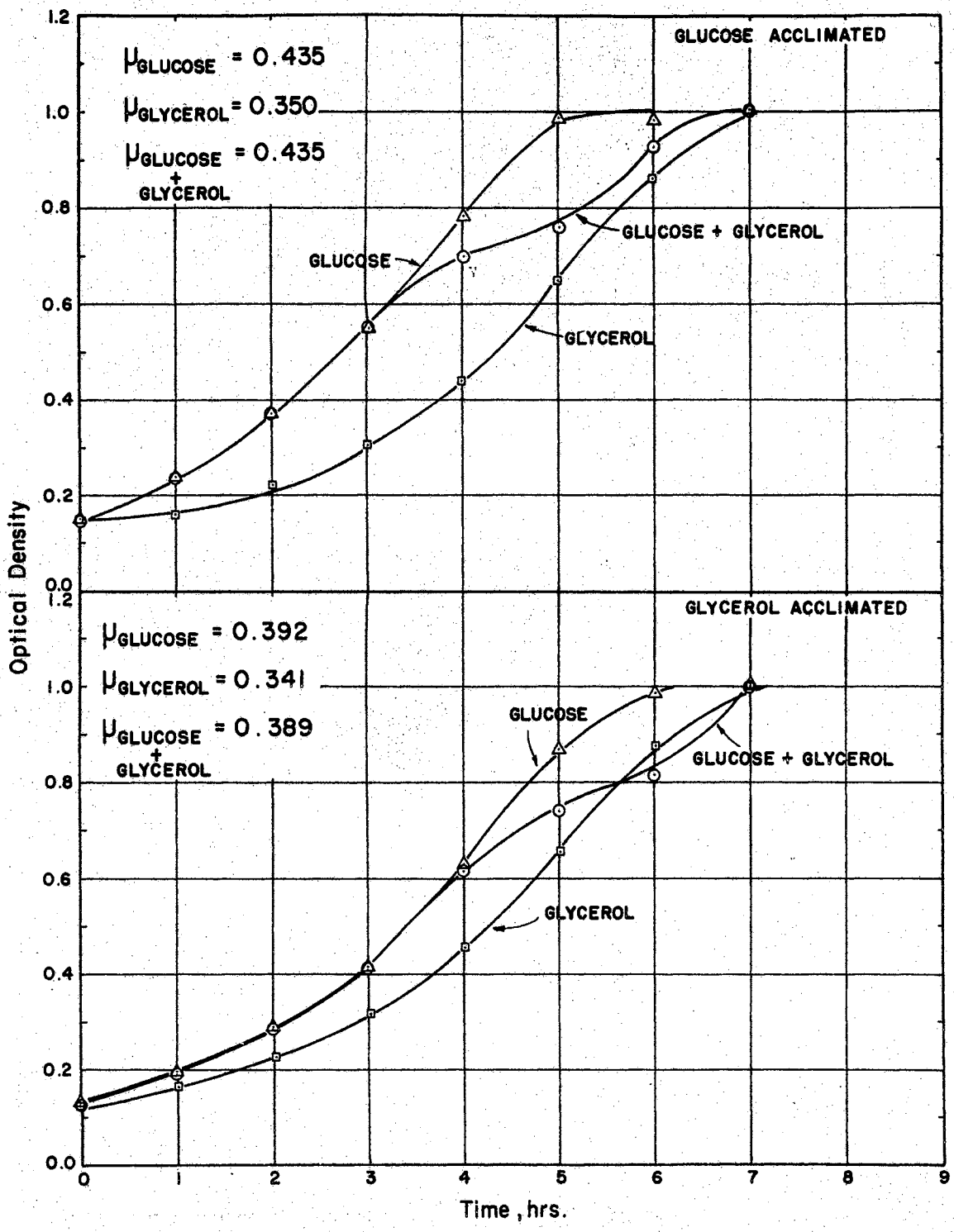

Figure 31. Exponential growth rate comparison of Blue organism on glucose and glycerol.

the acclimation history of the cells.

In Figure 32 the growth of Escherichia intermedia in a glucose-glycerol system is shown. While the μ values for glucose and glucose-glycerol are very similar for both systems, the μ on glycerol in the system acclimated to glucose was slightly higher than for the system acclimated to glycerol. Growth plateaus were observed in both glucose-glycerol curves, indicating sequential substrate removal. A lag period was observed in the growth curve on glycerol for cells acclimated to glucose.

In Figure 33 the results of an experiment with Serratia marcescens grown on glucose and glycerol are presented. No plateau was observed in this experiment, and it was seen that an acclimation period was required for the glucose-acclimated organisms in the glycerol medium. In all cases, growth rates for glycerol-grown cells were higher than those for glucose-grown cells.

In Figure 34 the growth curves for Blue organism on glucose and galactose are shown. A growth plateau can be noted in both two-substrate curves. A short lag was discernible in the curve for galactose inoculated with glucose-acclimated seed. For both sets, growth rates on glucose and on the combined substrates were considerably higher than on galactose.

The results of a Blue organism system on glucose and sorbose are shown in Figure 35. In the glucose-acclimated system, it is noted that a long period of acclimation to

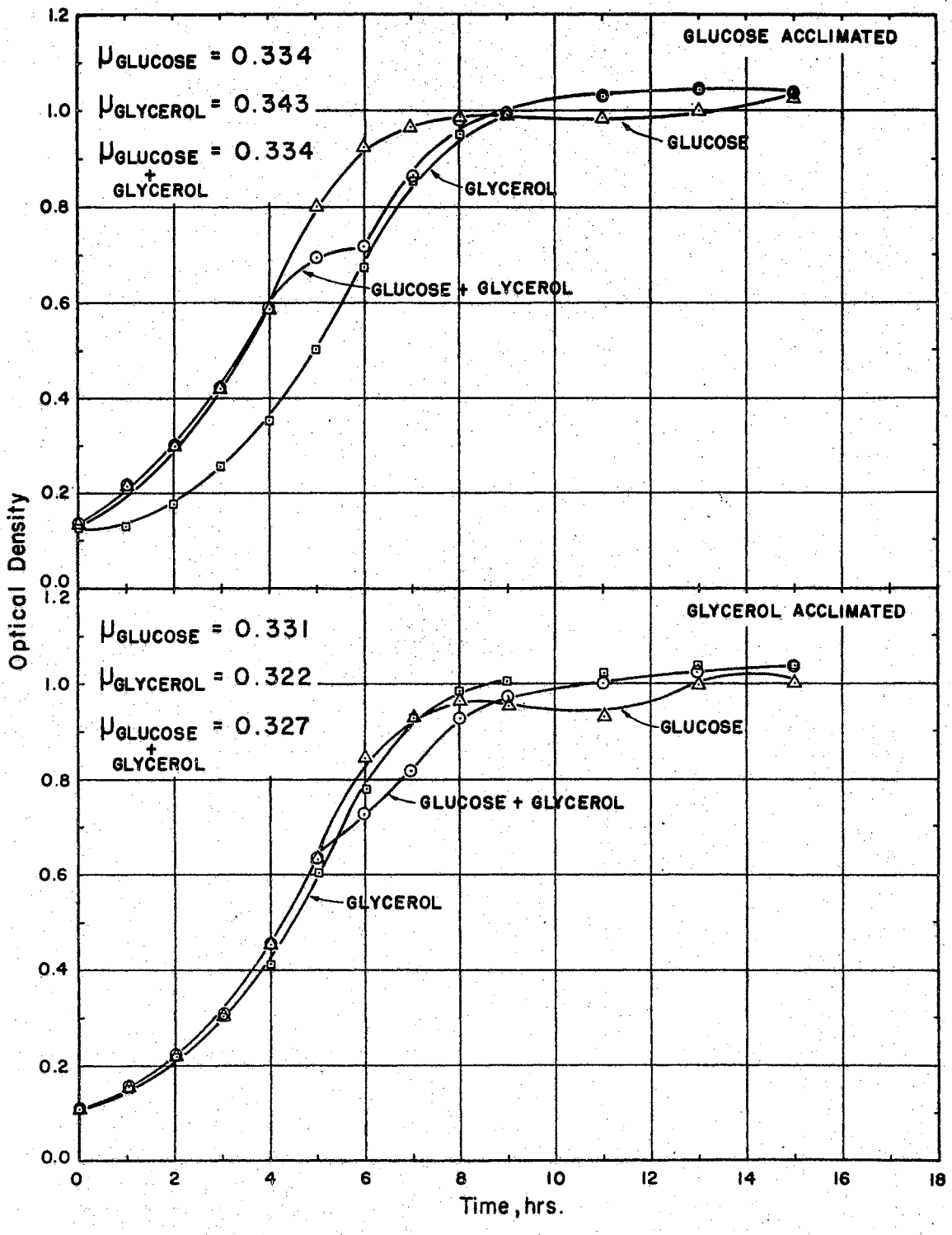

Figure 32. Exponential growth rate comparison of Escherichia intermedia on glucose and glycerol.

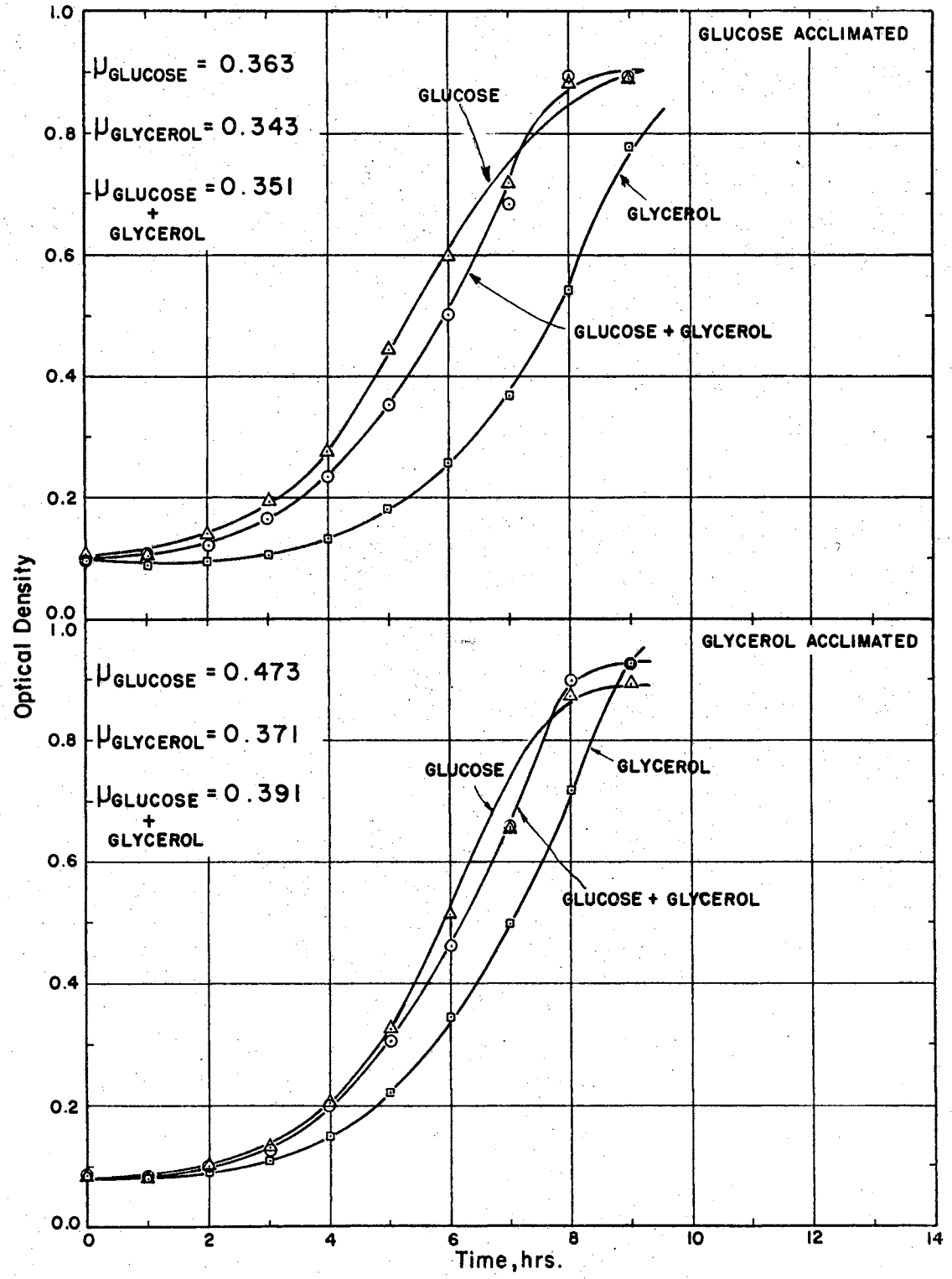

Figure 33. Exponential growth rate comparison of Serratia marcescens on glucose and glycerol.

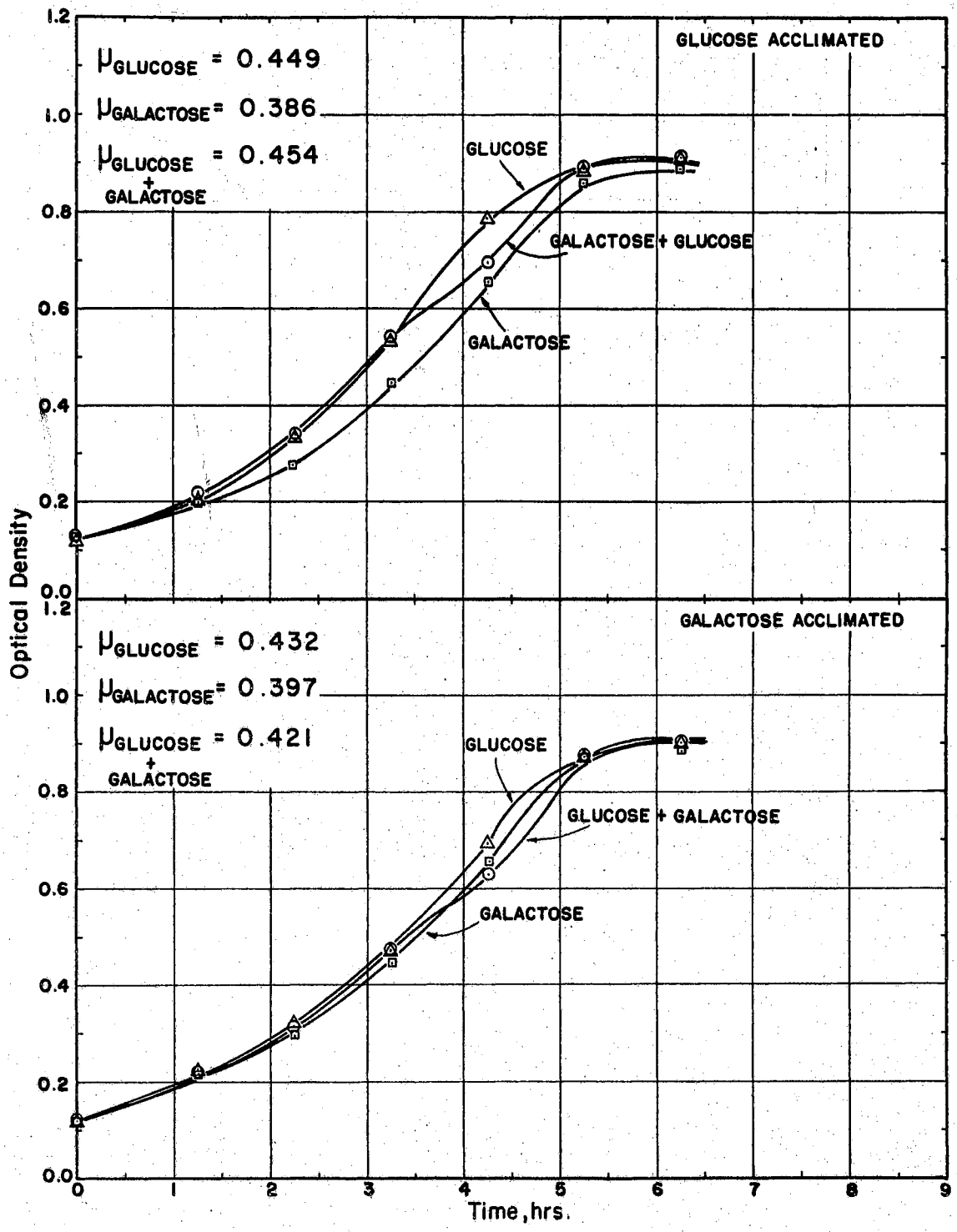

Figure 34. Exponential growth rate comparison of Blue organism on glucose and galactose.

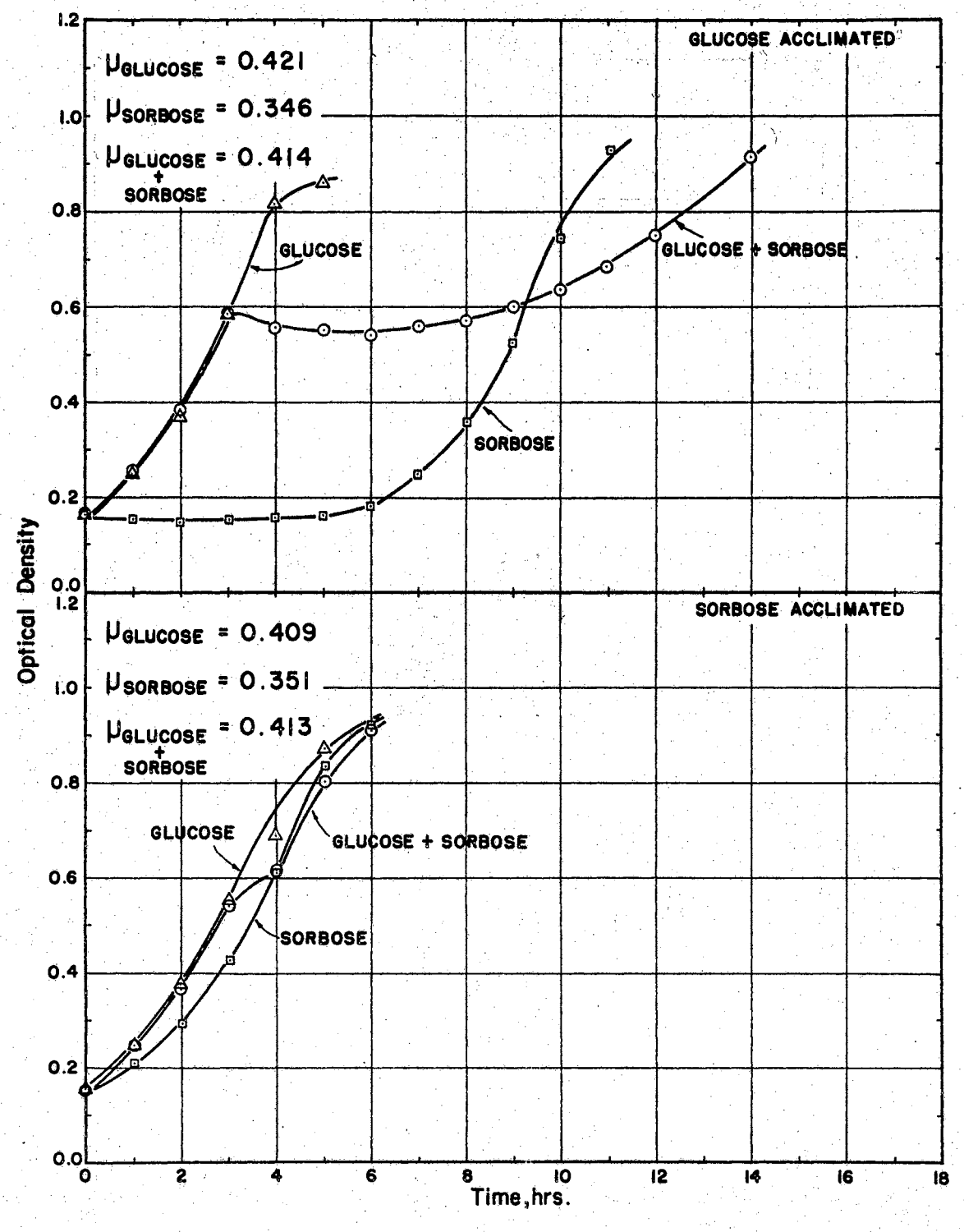

Figure 35. Exponential growth rate comparison of Blue organism on glucose and sorbose.

sorbose was required. Also, an extensive growth plateau can be seen in the two-substrate curve. Only a short growth plateau was observed in the two-substrate system for sorbose-acclimated organisms. As with galactose, growth rates on glucose or glucose-sorbose were considerably higher than on sorbose, regardless of the previous history of the cells.

In Figure 36 results of an experiment using Blue organism with glucose and sucrose are presented. In this experiment, all μ values for the sucrose-acclimated system were higher than those of the glucose-acclimated system. A growth plateau was observed in the two-substrate curve with glucose-acclimated seed, and a small lag period was detected in the sucrose curve. No plateaus or lags were noted in the sucrose-acclimated set, and all growth rates were essentially the same.

Results obtained with Serratia marcescens on glucose and sucrose are shown in Figure 37. The μ values of the sucrose-acclimated systems were slightly higher than those for the glucose-acclimated set. No plateaus were observed, although a lag in the sucrose curve for glucose-acclimated cells was noted.

The growth of Blue organism on glucose and ribose is shown in Figure 38. A prolonged growth lag was observed in ribose for the glucose-acclimated cells. Growth plateaus were observed in both two-substrate systems, although the plateau was much more marked with the glucose-

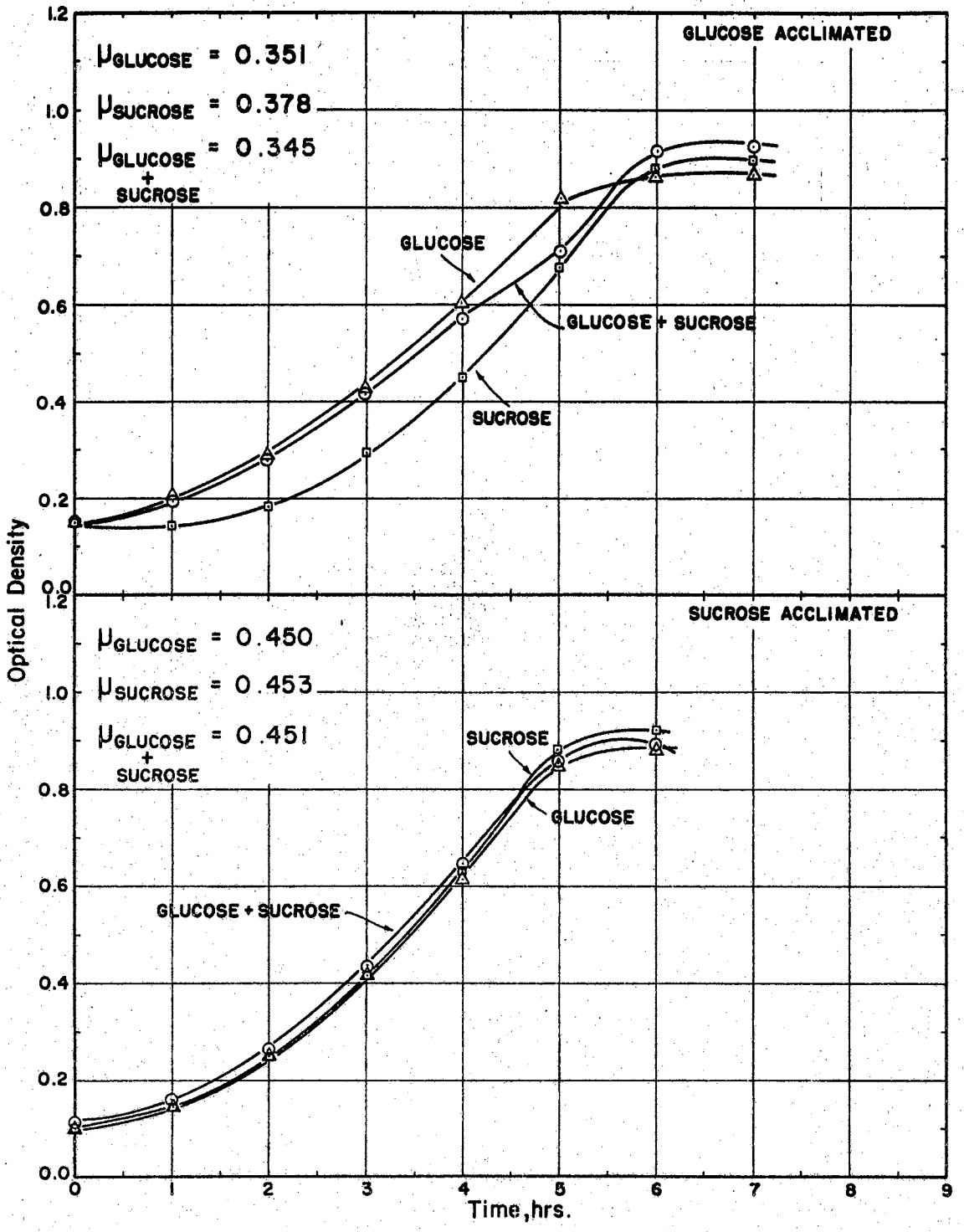

Figure 36. Exponential growth rate comparison of Blue organism on glucose and sucrose.

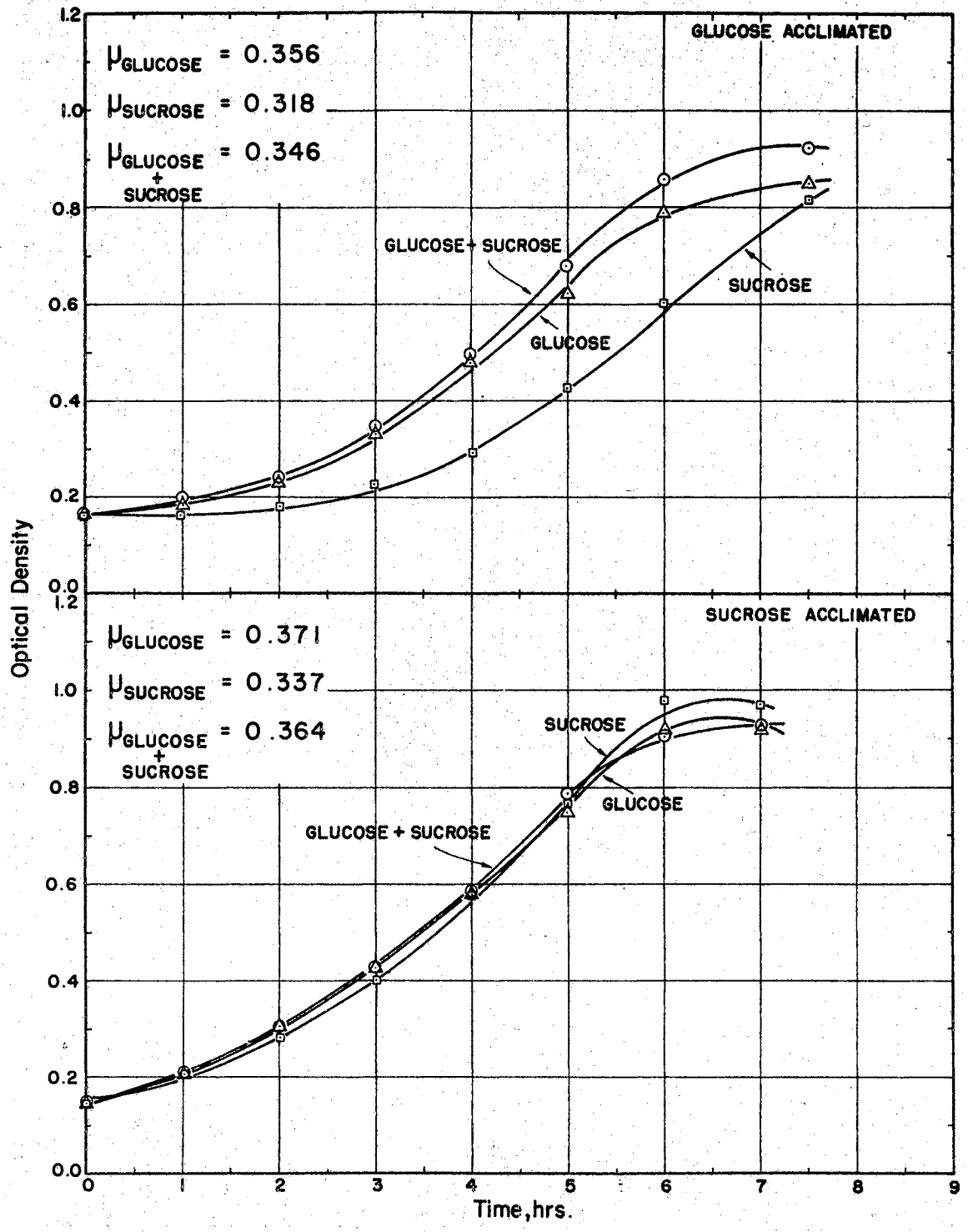

Figure 37. Exponential growth rate comparison of Serratia marcescens on glucose and sucrose.

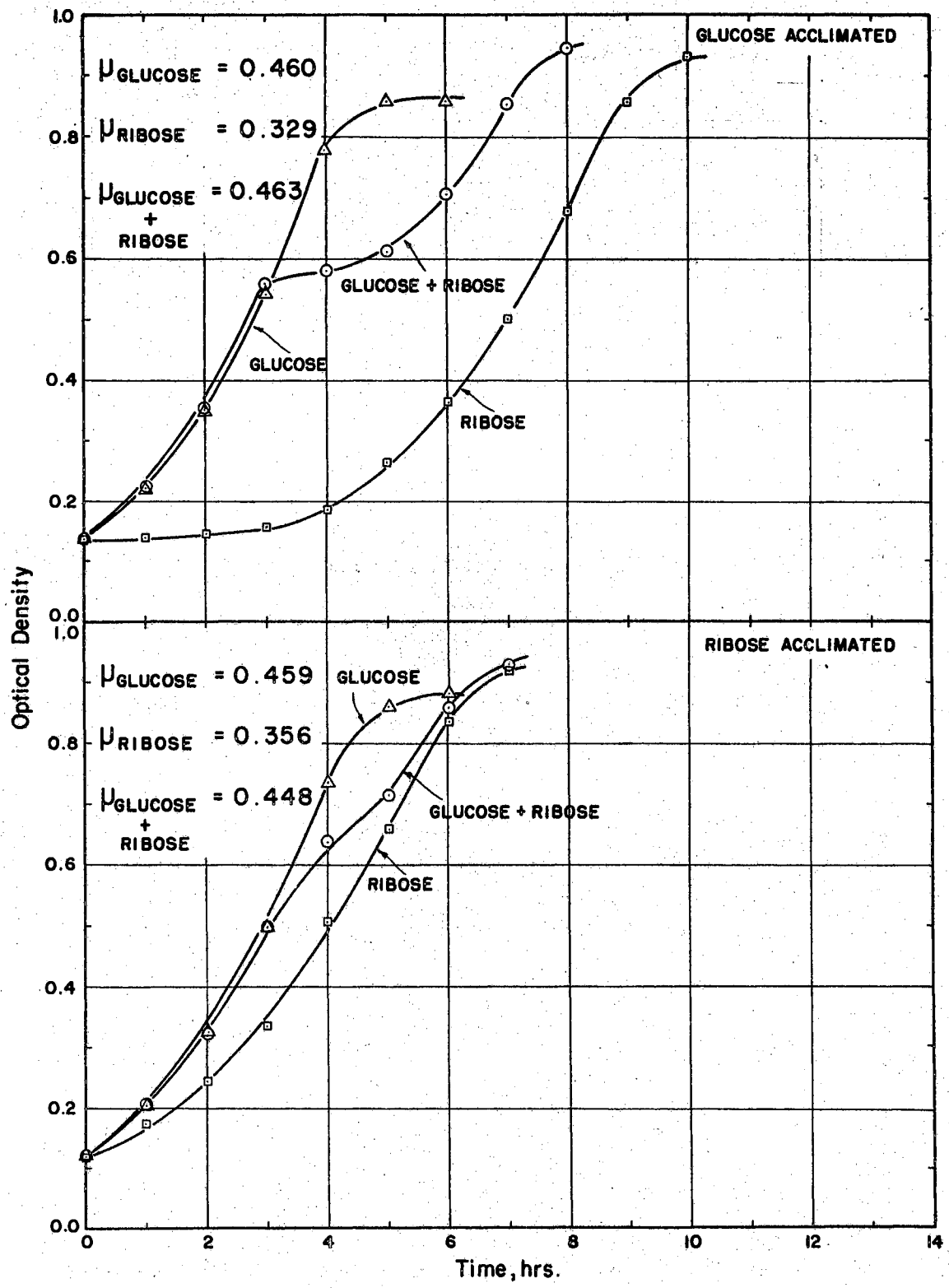

Figure 38. Exponential growth rate comparison of Blue organism on glucose and ribose.

acclimated cells.

In Figure 39 the growth of Serratia marcescens on glucose and ribose is presented. This organism grew at a much slower rate on ribose than on glucose. A lag was noted in the growth on ribose with the glucose-acclimated seed. A very slight plateau was observed in the two-substrate curve seeded with glucose-acclimated microorganisms. Ribose-acclimated cells grew more rapidly on glucose than did glucose-acclimated cells.

Results for a system in which Blue organism was grown on sorbitol and sucrose are presented in Figure 40. A short lag was observed in both single substrate curves for non-acclimated cells. A plateau was observed in the two-substrate curve for the sucrose-acclimated system.

Figure 41 shows the growth curves for Blue organism in the presence of fructose and ribose. Growth rates for all three parallel cases were very similar. The two-substrate system exhibited a plateau when seeded with fructose-acclimated cells. A lag was noted in the ribose curve with fructose-acclimated organisms, and it is noted that fructose was used more readily by ribose-acclimated cells than was ribose.

Figure 42 shows the results of an experiment using sucrose and mannitol as substrates. These curves show no appreciable plateaus or lags and in general, the growth rate on the combined system was nearly the same as that observed on the single carbon source to which the cells were acclimated.

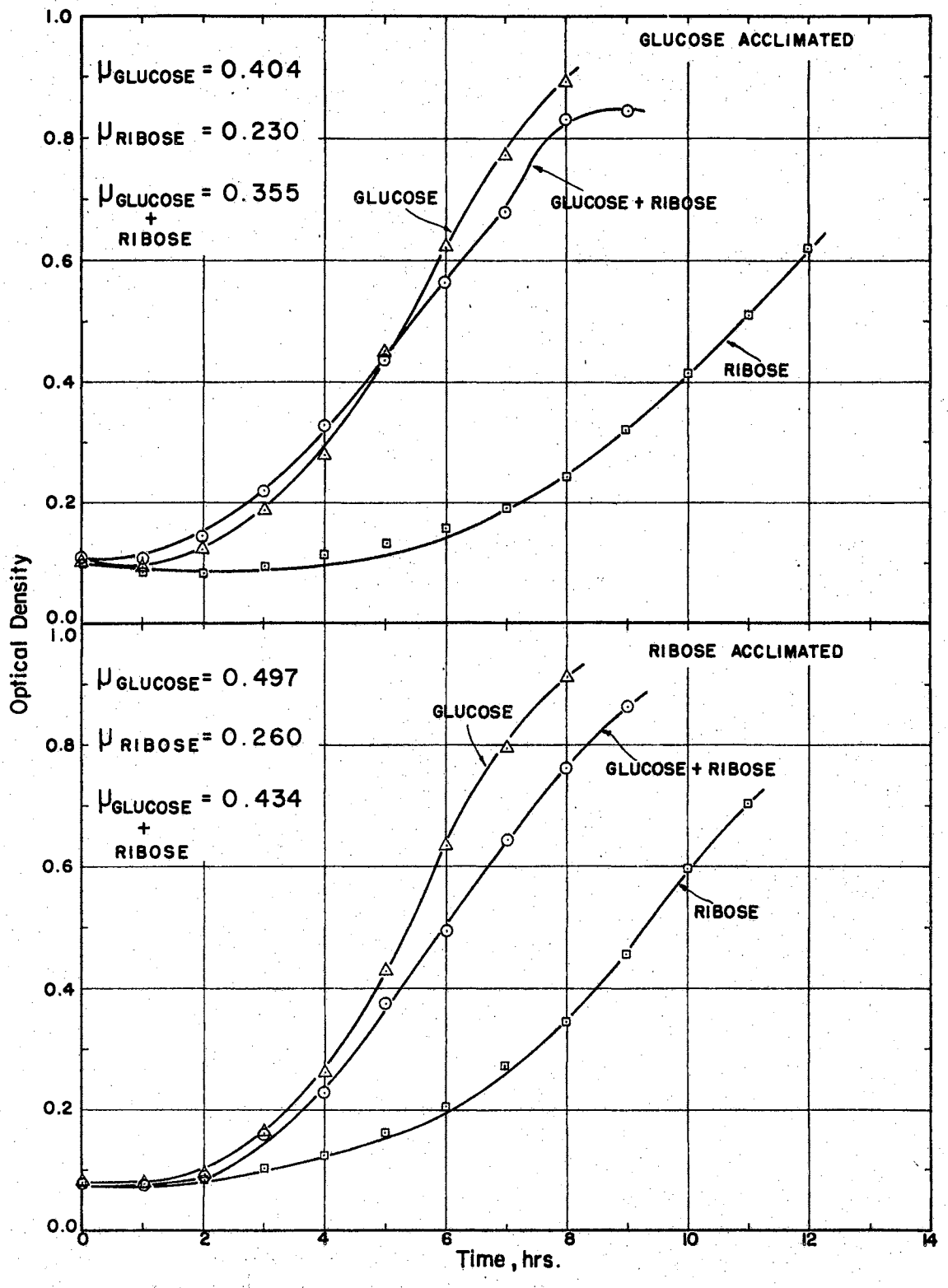

Figure 39. Exponential growth rate comparison of Serratia marcescens on glucose and ribose.

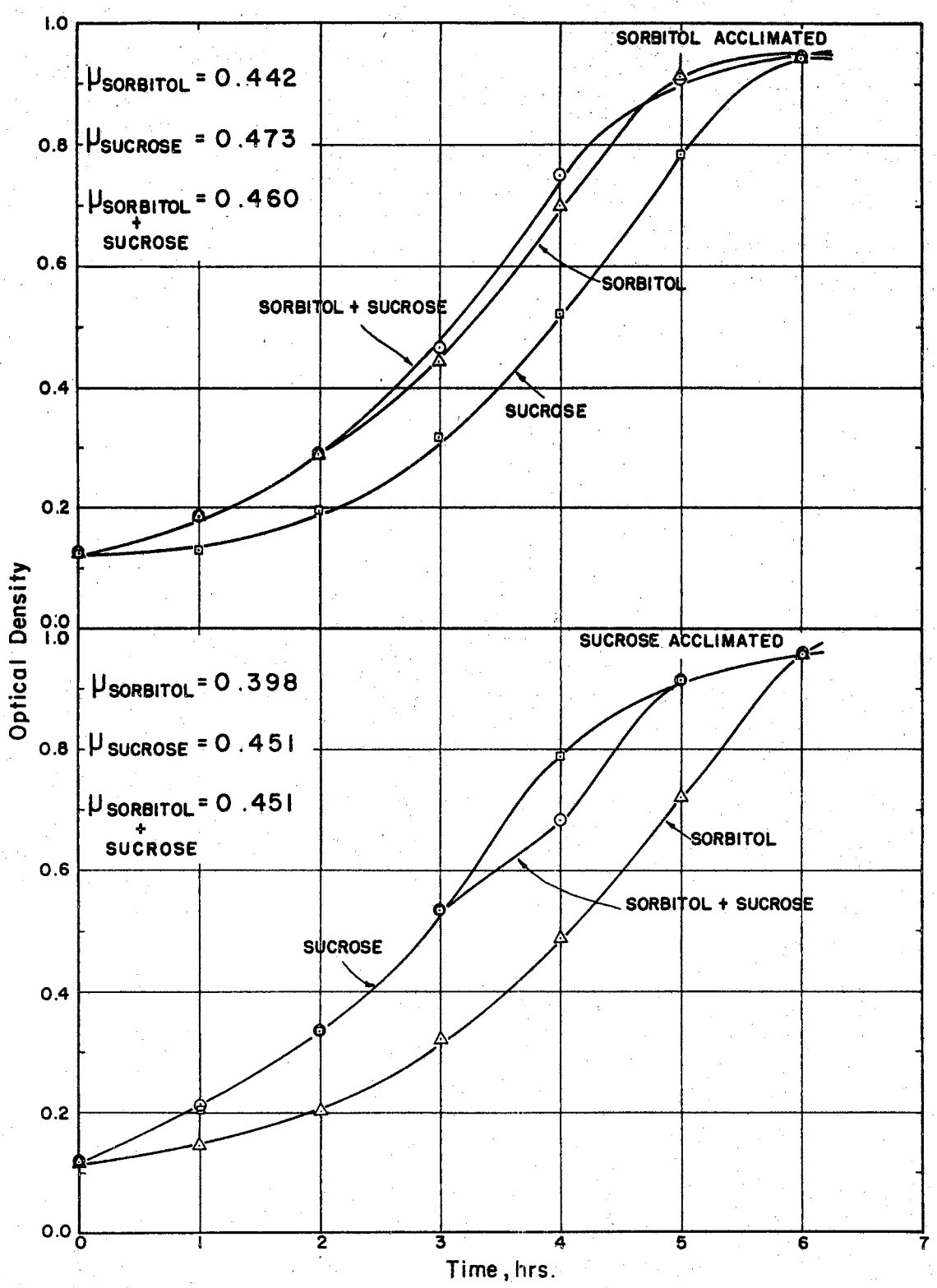

Figure 40. Exponential growth rate comparison of Blue organism in sorbitol and sucrose.

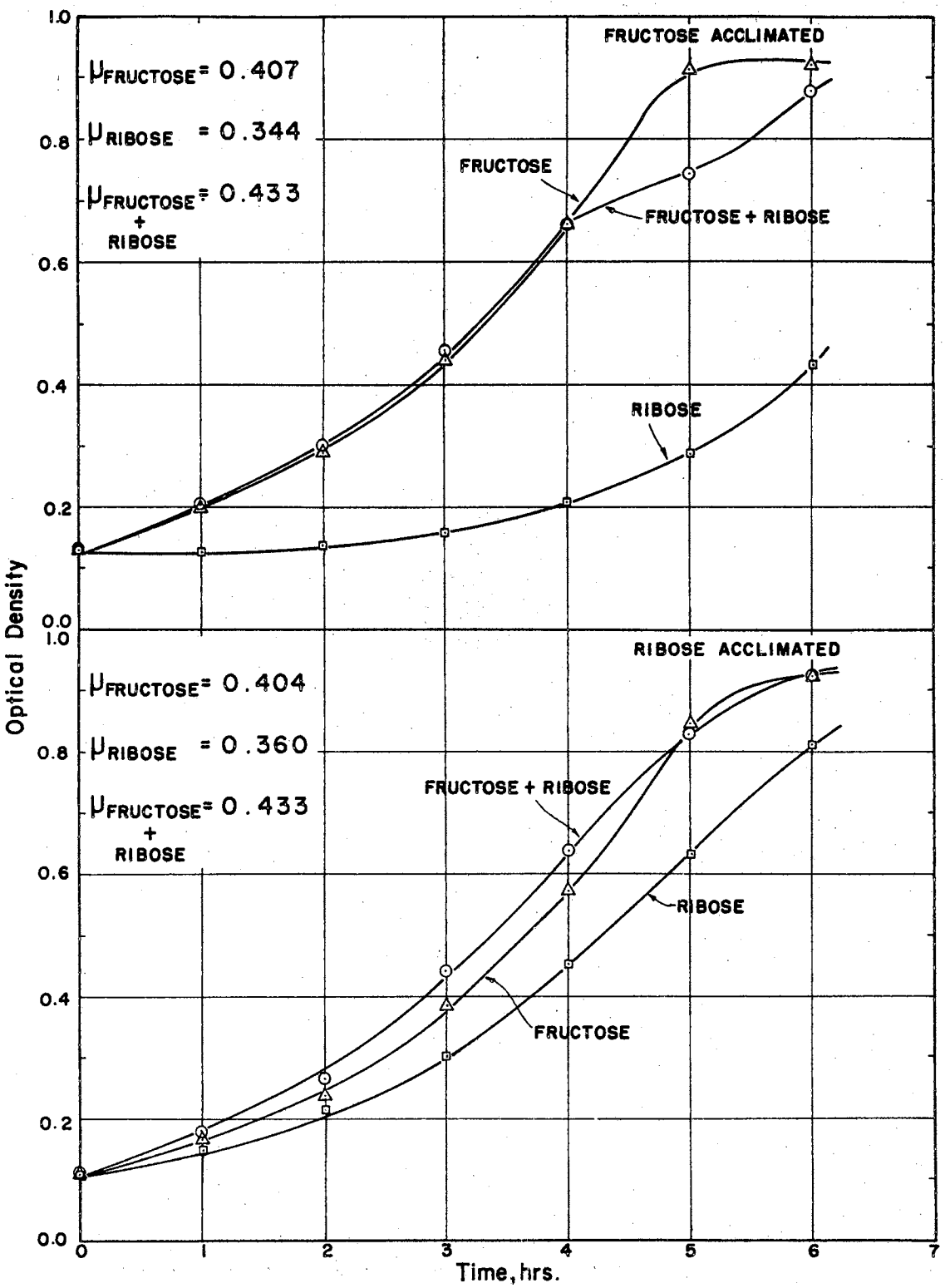

Figure 41. Exponential growth rate comparison of Blue organism on fructose and ribose.

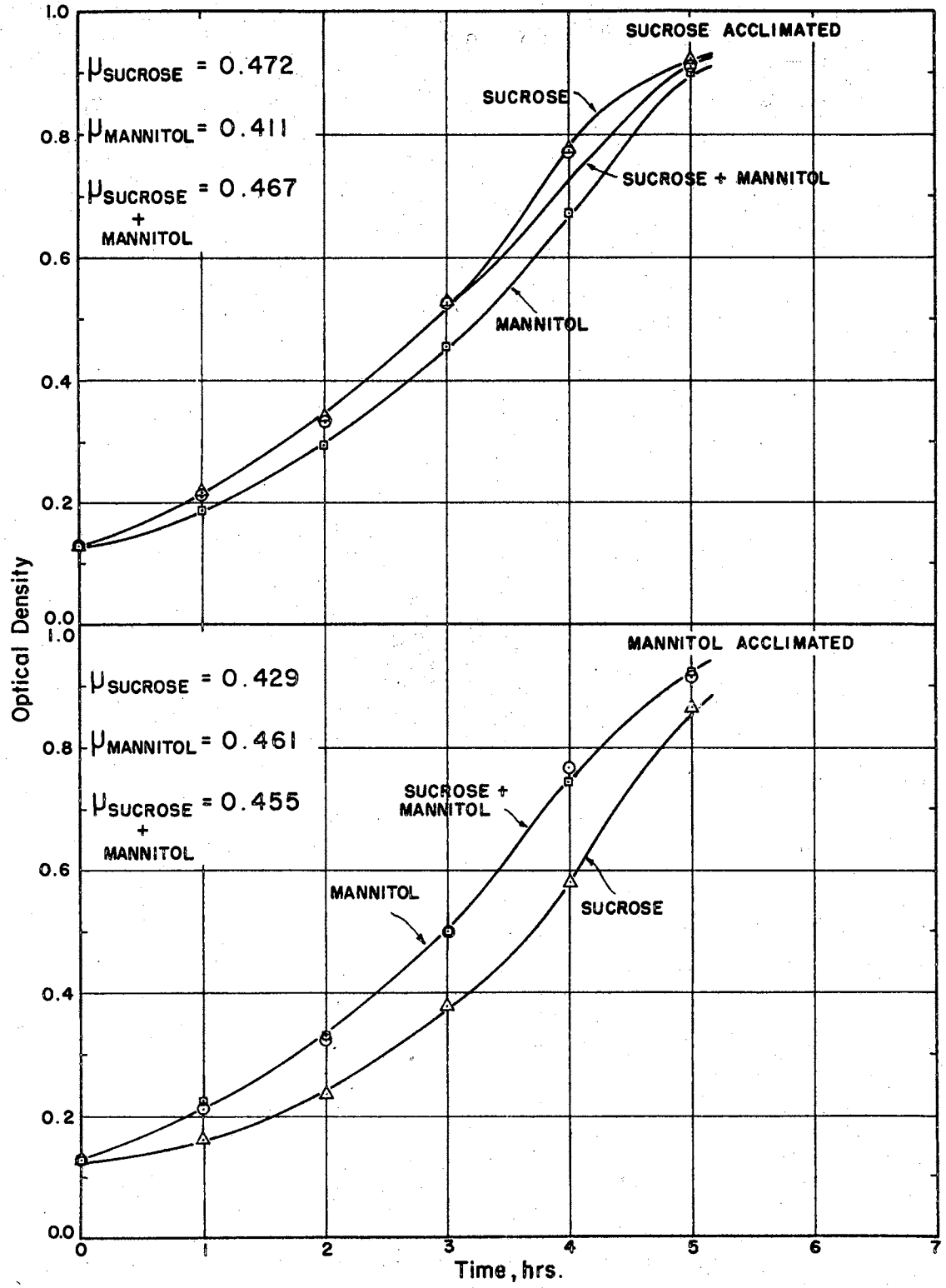

Figure 42. Exponential growth rate comparison of Blue organism on sucrose and mannitol.

Analysis

The previous history of a population of cells might conceivably have several effects upon subsequent growth of the population when placed in new medium. Cells grown on a substrate requiring inducible enzymes should be able to utilize that substrate without a lag and, depending upon the amount of substrate available and the proportion consumed before maximum induction is achieved, might reach a higher growth rate than could be reached by non-acclimated cells. Prior growth on a closely related substrate might shorten the lag period if fewer enzymes needed to be induced. Prior growth on a related compound also might lead to more rapid growth on a new substrate which could be utilized through two pathways, one induced by the previous substrate. Cells placed in mixtures of substrates after prior growth on one of the substrates may be expected to behave quite differently, depending upon which substrate had been previously used and whether one substrate is capable of repressing synthesis of enzymes required to utilize the other. Several of these effects were noted in the experiments just described.

Lag periods in growth curves for acclimated and non-acclimated seeds may be compared as an indication of constitutivity or inducibility of enzyme systems. Glucose was used constitutively, i.e., without a lag, by all three organisms regardless of the previous history of the cells. Neither galactose, fructose, nor mannitol was subject to a

significant lag in growth when non-acclimated cells were used, although in all three cases growth was slightly more rapid with an acclimated inoculum (Figures 34, 41, and 42). It might be concluded from the data obtained that enzymes for utilization of these carbon sources are very rapidly induced. In contrast, both ribose and sorbose were used only after very long periods, approximately five hours for sorbose with Blue organism (Figure 35), three hours for ribose with Blue organism (Figure 38), and three to four hours for ribose with Serratia marcescens. Enzymes for utilization of these carbon sources are therefore not rapidly induced and, in the case of Serratia marcescens at least, growth on ribose continued to be quite slow. Lag periods with non-acclimated cells were observed for sorbitol, glycerol, and sucrose, with all three organisms. These were generally less pronounced than those for ribose or sorbose, and varied with the organisms used.

A diauxic lag, or diphasic growth curve, was observed in many of the two-substrate systems (Figures 28, 29, 31, 32, 34, 35, 36, 38, 39, 40, 41). This type of growth curve is an indication of sequential substrate removal which has been described for heterogeneous populations by Gaudy, Komolrit, and Bhatla (109), and by Gaudy, Gaudy and Komolrit (110) for Escherichia coli. However, it has been shown by these authors that substrates may be removed sequentially without detectible effects upon growth kinetics. Since substrate removal was not measured in this

set of experiments, only evidence based on growth kinetics can be considered. In all cases where diphasic growth occurred, previous acclimation of the cells to the substrate which was presumably subject to repression affected the severity of the disruption of growth. In several cases the diauxie in growth was completely eliminated. These included: glucose-sorbitol with Escherichia intermedia, glucose-ribose with Serratia marcescens, and glucose-sucrose, sucrose-sorbitol, and fructose-ribose with Blue organism. In other cases, the diauxic lag was decreased but not eliminated. These included: glucose-glycerol with Escherichia intermedia, glucose-sorbitol, glucose-glycerol, glucose-galactose, glucose-sorbose, and glucose-ribose with Blue organism. In the latter two systems a very prolonged lag occurred with glucose-acclimated cells and the lag was reduced drastically by growth of the inoculum on sorbose and ribose, respectively. These results indicate that induced enzymes can be retained during periods when synthesis of new enzyme is repressed. If the period of repression is not prolonged until complete dilute-out has occurred, initiation of growth on the previous carbon source can be greatly facilitated.

In general, μ values for these experiments varied within the series of experiments and in comparison to values reported in Tables II and IV. Since the present set of experiments was not designed specifically for measurement of kinetic constants, this variation may possibly be

explained on the basis that the μ values obtained were not μ_{max} values; i.e., substrate may have limited during parts of the experiments. Also, some variations in growth rates for single cultures were noted in experiments separated by considerable time intervals. This was attributed to possible mutations of the cultures. However, within a single experiment, in which single populations were used as inoculum, valid comparisons of growth rates can be made.

The lowest growth rate obtained was that for Serratia marcescens on ribose. The cells used in this experiment were capable of very rapid growth, as evidenced by their growth on glucose. It would appear that this organism simply utilizes ribose at a slow rate and that the rate is increased only slightly by prior growth on ribose.

Other substrates which appeared to support slow growth, in comparison with that on other substrates used with the same population, were glycerol, galactose and sorbose in experiments with Blue organism. This organism also grew comparatively slowly on ribose.

Comparison of growth rates within experiments reveals both postulated effects of acclimation on μ. With Blue organism, μ values for acclimated cells were higher than those for non-acclimated cells with glucose, sucrose, sorbitol, and mannitol. This enhancement of growth rate by acclimation depends upon the identity of the second substrate of the pair, since it was observed with glucose only when compared to glycerol-grown cells and with sorbitol only

when compared to sucrose-grown cells. The observed differences are sufficiently large to be significant. It is possible that growth on certain substrates produces cells which are acclimated not only to that substrate, but also to a specific rate of growth which cannot immediately be adjusted to a new rate when the substrate is changed.

In three cases, growth rate on one substrate was enhanced by prior growth on a different substrate. This was observed for Serratia marcescens acclimated to glucose and placed in sorbitol, and for the same organism acclimated to ribose and placed in glucose. Both of these cases may indicate utilization by the new substrate of enzymes induced by the previous substrate. The effect was particularly noticeable in the glucose-ribose system, and may indicate increased use of the pentose shunt due to prior growth on ribose. In the third case, Blue organism, acclimated to sucrose and placed in glucose, may afford further support of the mechanism advanced above, i.e., the persistence of established growth rates. In this experiment (Figure 36) cells grown on sucrose had significantly higher μ's on both glucose and sucrose than did glucose-grown cells. However, the same organism, in an experiment using sucrose and sorbitol (Figure 40), did not grow as rapidly on sorbitol as on sucrose. In general, growth rates on sucrose were consistently higher than on any other substrate used, and prior growth on sucrose may tend to increase growth rates on substrates which can be utilized without a

significant lag.

6.3 Determination of Equivalent Operational Conditions for the Shaker, Warburg Apparatus and Chemostat

In the many experiments performed in connection with this work, it was necessary and desirable, at times, to incubate cultures under different agitation and aeration conditions. A series of experiments was designed to determine the rates of growth and substrate removal on the shaker flask apparatus, in the Warburg apparatus and in the chemostat in order to find conditions which would be closely equivalent regardless of the apparatus used in a particular experiment. Pure cultures of bacteria were used as test organisms, and optical density measurements, biological solids production, and COD removal were used to assess the behavior of the test organism under the various experimental conditions. All experiments were carried out at a constant temperature of 25°C.

In experiments involving the water bath-shaker and the Warburg apparatus, it was found that the behavior of the test organisms in both systems was very similar when the shaker was operated at 110 oscillations per minute. In comparing the performance of the shaker and the chemostat, operated as a batch system, it was determined that both types of equipment yielded comparable results when the shaker was operated at 90 strokes per minute and the chemostat aerated at a rate of 1000 cc per minute. In all experiments herein reported, the equivalent rates of

agitation and aeration determined were utilized.

7. Biochemical Behavior of Pure Cultures on Glucose and Sorbitol

In these experiments, COD removal, oxygen uptake, biological solids concentrations, and substrate removal were used as parameters to assess the biochemical behavior of the pure cultures studied under growth conditions using glucose or sorbitol as sole carbon source. Figure 43 shows the biochemical behavior of Blue organism using glucose as the carbon source. The organism grew very rapidly, as can be seen from the solids curve. In like manner, the COD and glucose COD curves show that the substrate was removed from the medium at a fairly rapid rate. Also noted from these results is the fact that considerable amounts of intermediates and/or end products were produced during the glucose removal period. Such intermediate production may be discerned by comparing the COD curve with the glucose COD utilization curve.

Results for an experiment in which Pseudomonas aeruginosa was used employing glucose as substrate are presented in Figure 44. Rapid COD and glucose COD removals were noted in this experiment, and comparison of the two curves shows that large amounts of metabolic intermediates were produced. The biological solids curve parallels the oxygen uptake curve.

In Figure 45, results for an experiment using Escherichia intermedia with glucose as substrate are

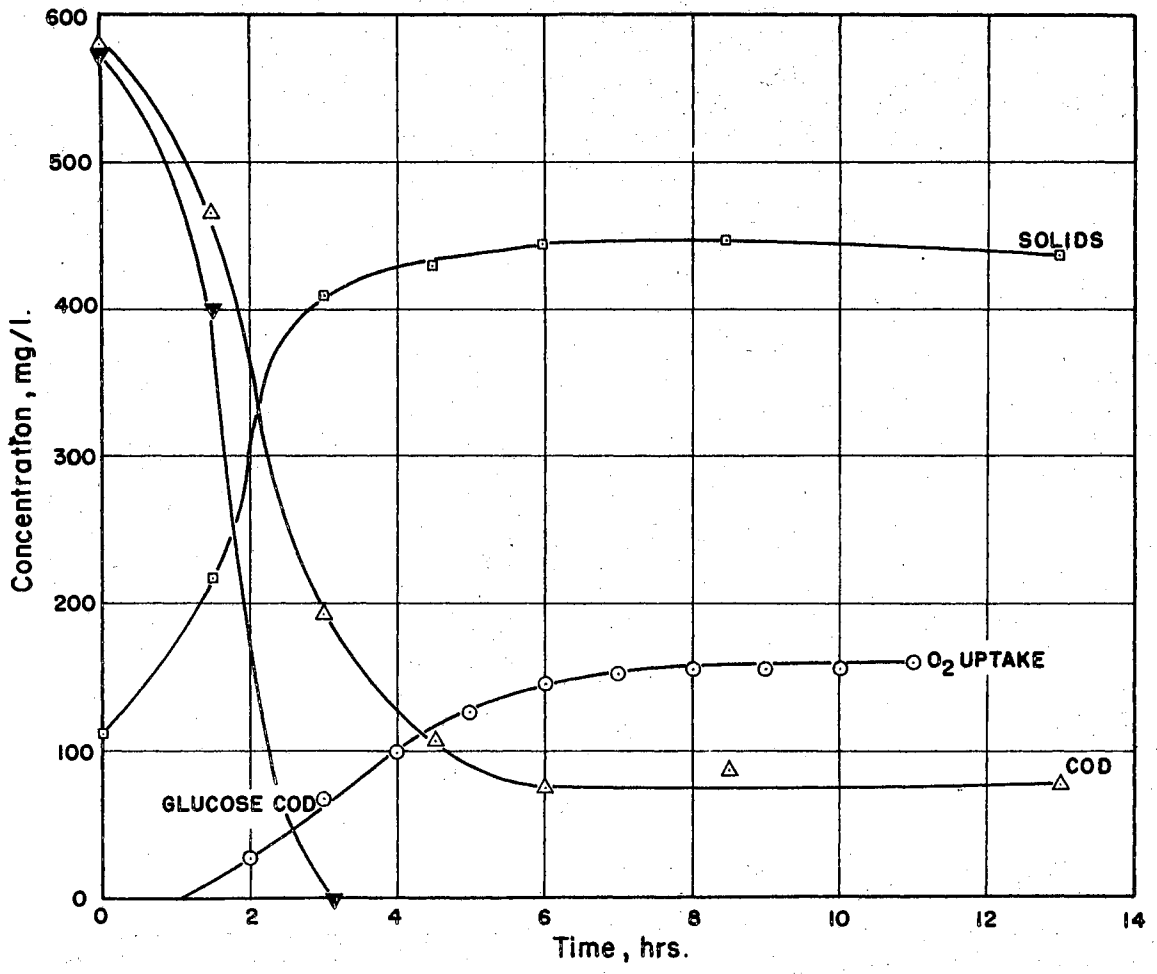

Figure 43. Substrate removal and growth of Blue organism.

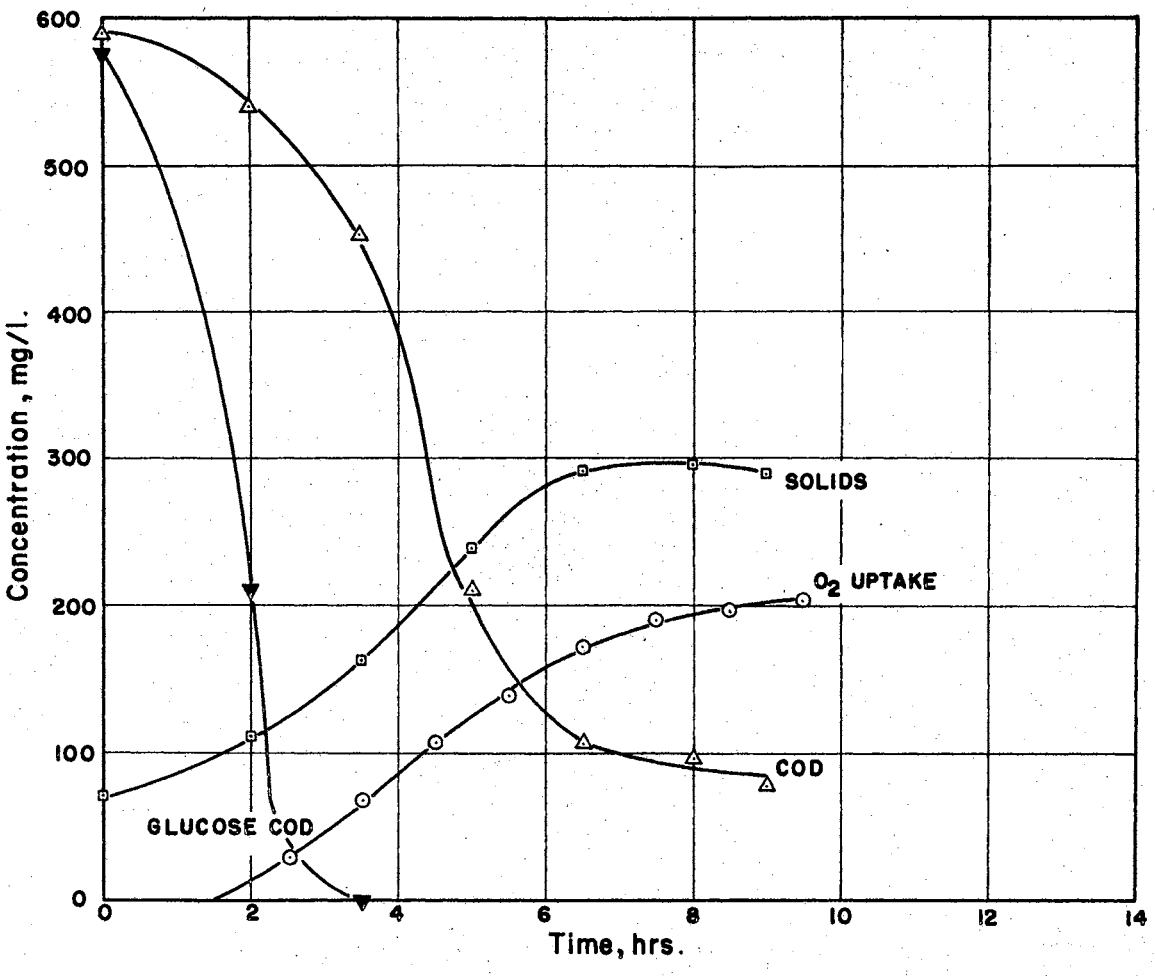

Figure 44. Substrate removal and growth of Pseudomonas aeruginosa.

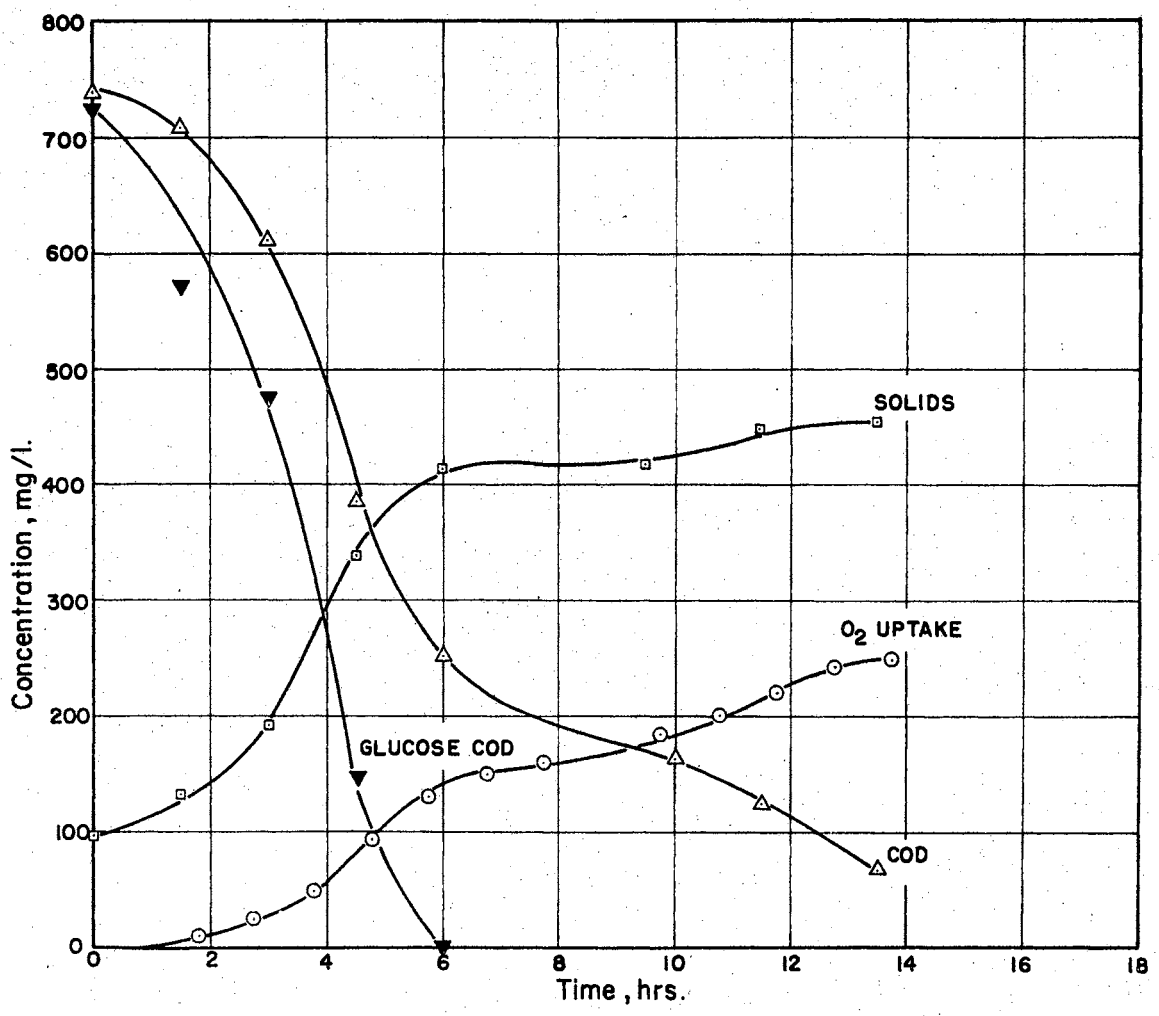

Figure 45. Substrate removal and growth of Escherichia intermedia.

presented. In this experiment, considerable amounts of intermediates were produced. The oxygen uptake curve shows the plateau that is characteristic of this organism. Plateaus were also noted in the biological solids curve and the COD removal curve. The same plateau effect in optical density measurements was also noted during the experiment, but is not shown in the figure. The beginning of the plateau effect, as noted in the solids, COD and oxygen uptake curves, coincided with the elimination from the medium of the glucose furnished as substrate.

From Figure 46 it can be seen that the organism Serratia marcescens produced considerable amounts of metabolic intermediates and/or end products during the substrate removal period, when glucose was used as substrate. Examination of Figure 47 shows that a very limited amount of material was excreted into the medium during growth by Yellow organism on glucose.

Results of an experiment using glucose as substrate and Escherichia coli as test organism are shown in Figure 48. It can be seen that this organism grew very slowly. Comparison of the COD and the glucose COD removal curves indicates that Escherichia coli produced metabolic intermediates and/or end products, although to a lesser extent than did Blue organism, Pseudomonas aeruginosa, Excherichia intermedia, or Serratia marcescens.

Figure 49 shows the biochemical behavior of Blue organism growing on sorbitol. Examination of the COD

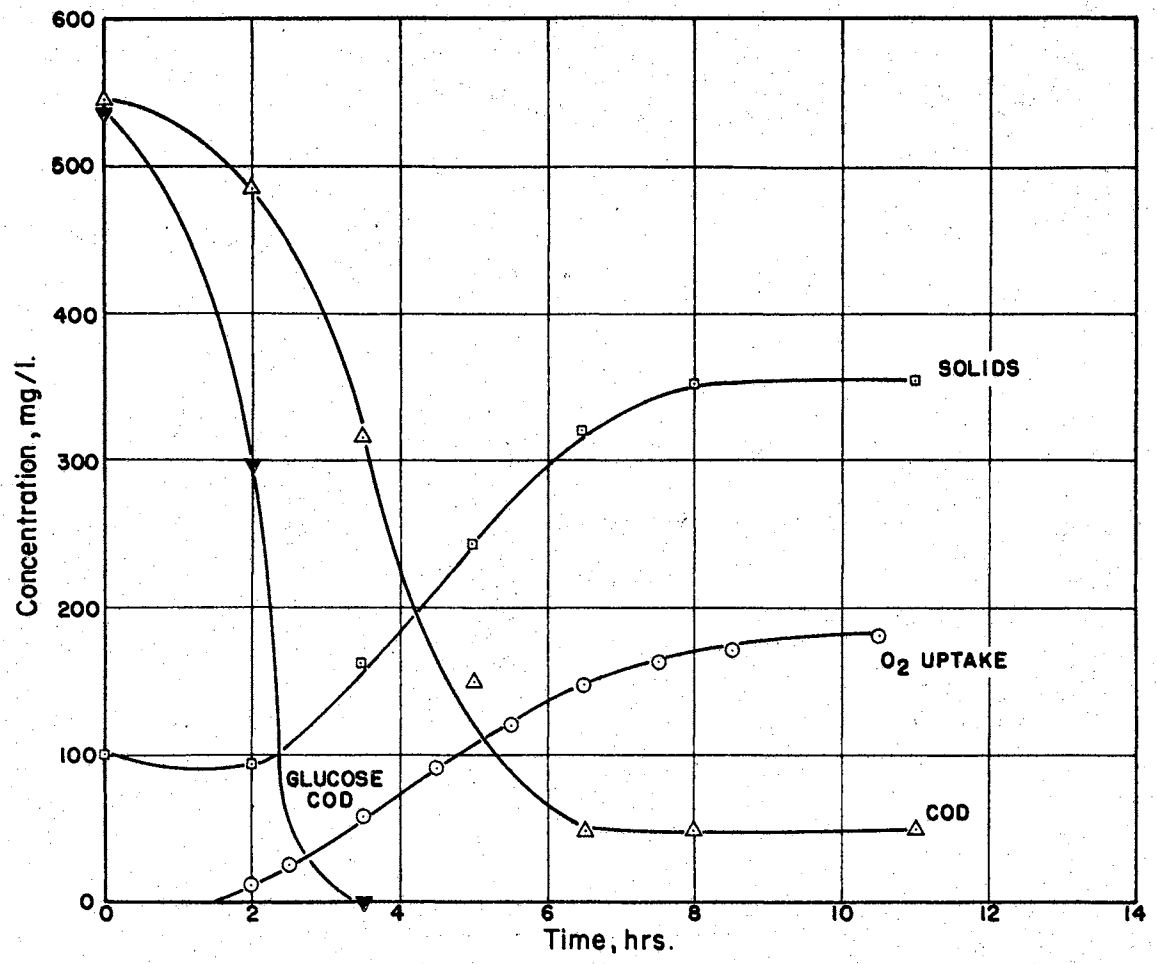

Figure 46. Substrate removal and growth of Serratia marcescens.

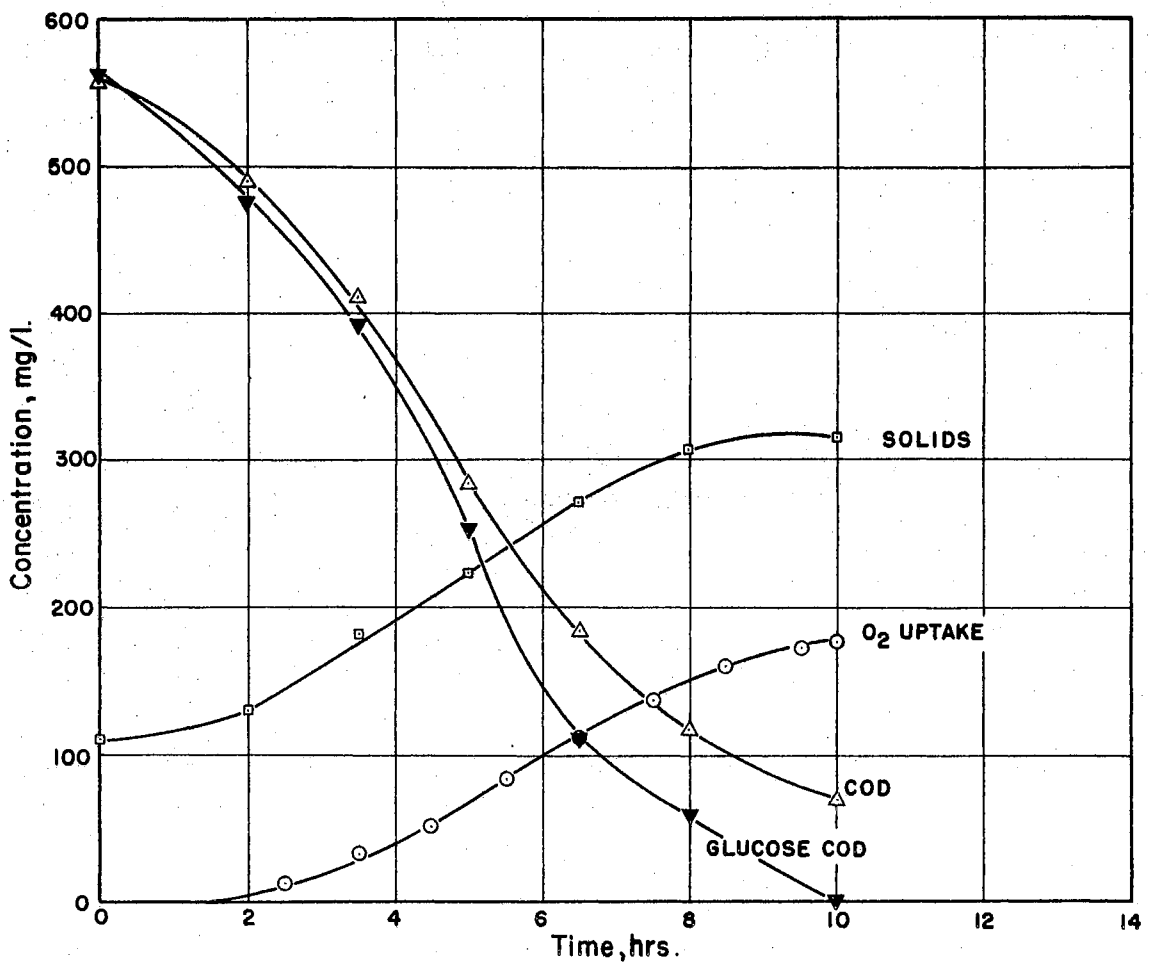

Figure 47. Substrate removal and growth of Yellow organism.

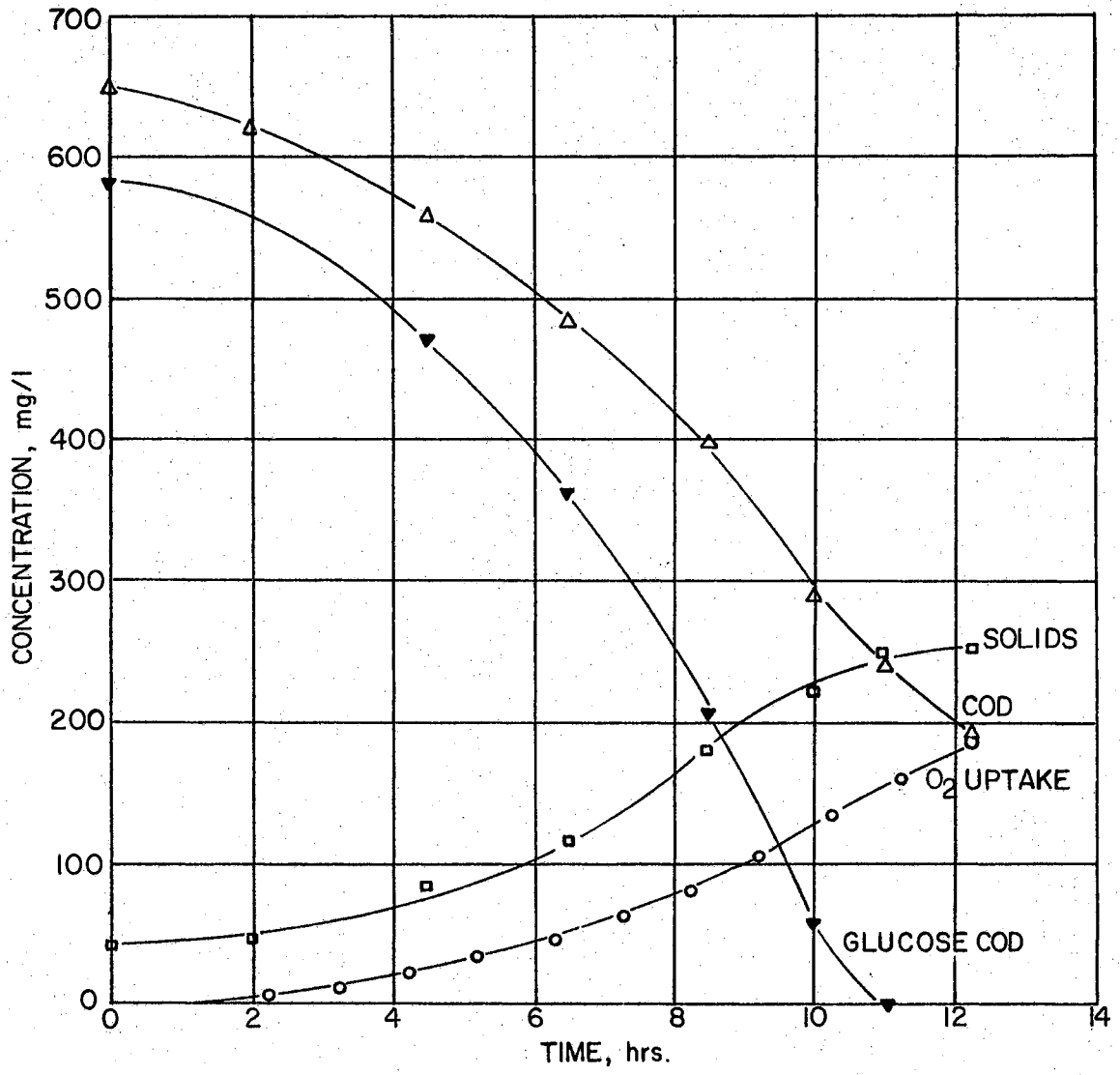

Figure 48. Substrate removal and growth of Escherichia coli.

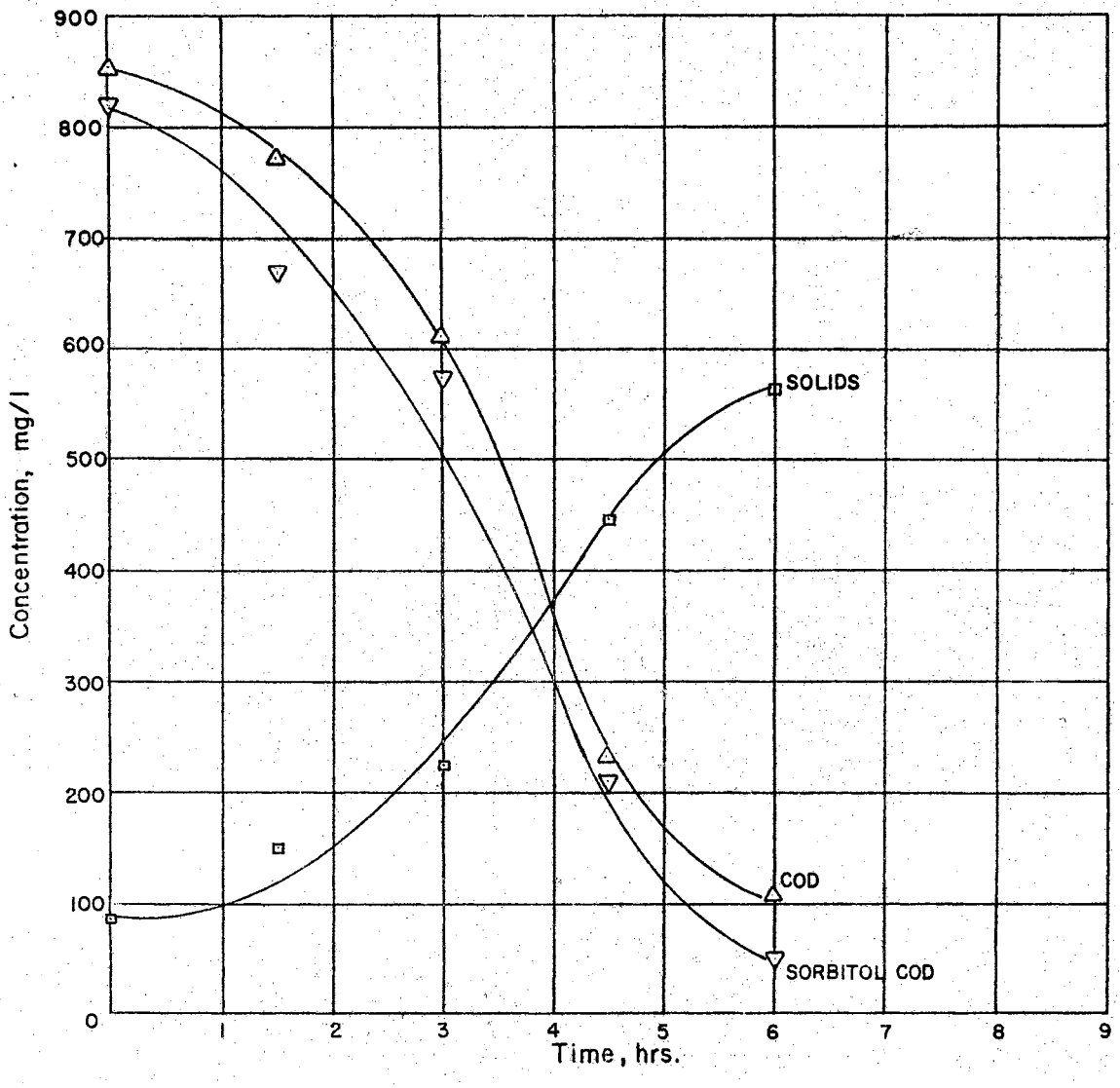

Figure 49. Sorbitol removal and growth of Blue organism.

removal and the sorbitol COD utilization curves shows that little or no intermediate accumulation occurred.

Results of an experiment using sorbitol as substrate and Serratia marcescens as test organism are shown in Figure 50. These results were very similar to those shown in Figure 49 in that no metabolic intermediates were produced.

Analysis

Examination of Figures 43-48 indicates that Blue organism, Pseudomonas aeruginosa, Escherichia intermedia, and Serratia marcescens introduced large amounts of metabolic intermediates into the medium when incubated in the presence of glucose. In regard to the classification of organisms in accordance with their μ_{max} values presented earlier, it can be seen that three of the four organisms mentioned above were included among the fast growers. The fourth organism, Serratia marcescens, was classified as a medium-fast grower. From Figures 47 and 48 it can be seen that Yellow organism and Escherichia coli were less active in the production of intermediates; Yellow organism had previously been classified as a medium-fast grower and Escherichia coli had been classified as a slow grower. It is interesting to note that the fast growers produced more intermediates than the slow growers; these observations suggest a possible relationship between the μ_{max} of an organism and its ability to take up glucose at a fast rate while introducing relatively large amounts of metabolic

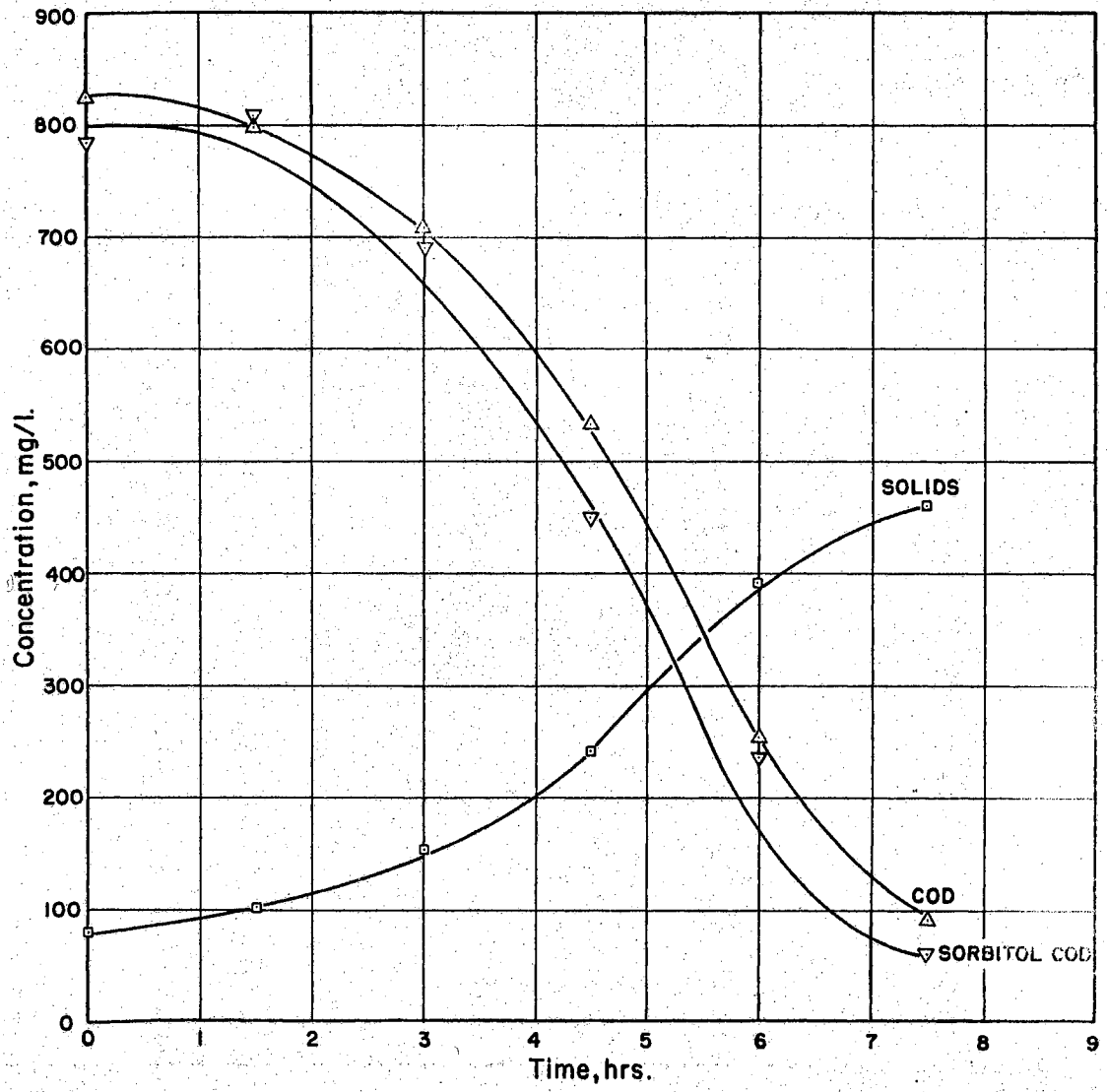

Figure 50. Sorbitol removal and growth of Serratia marcescens.

intermediates into the medium. Such a relationship might work to the disadvantage of the fast grower if it were growing together with a slow grower. However, there is the case of Yellow organism (Figure 47) where very few intermediates were produced although the organism was classified as a medium-fast grower, thus indicating that the μ_{max} intermediates relationship may not be applicable to every organism that is capable of utilizing glucose as a sole source of carbon. The possibility of bacterial interaction in mixed systems through metabolic intermediates is explored later in this report.

From Figures 43, 45, and 46 it can be surmised that the metabolic intermediates introduced into the medium were rapidly metabolized after the glucose was exhausted. This indicates the presence in the cells of the requisite enzymes for the assimilation of the intermediate products. These enzymes may have been constitutive or, if they were inducible, they were apparently synthesized while glucose was still present in the medium. In any event, there was no discernible acclimation period. An exception to the usual behavior of the cultures is noted in the case of Escherichia intermedia (Figure 45), where the cells were unable to assimilate the intermediate products without a lengthy acclimation period as evidenced by the diphasic nature of the COD, biological solids, and oxygen uptake curves. It is surmised that the plateau observed between the two phases of metabolic activity represents the time

necessary for the cells to acclimate to the metabolic products. The particular behavior of Escherichia intermedia, which has been previously reported for this and other organisms (107)(111)(112), may be explained by the fact that the organism may not possess constitutive enzyme for the assimilation of the intermediates and that a considerable amount of time was needed for the synthesis of these enzymes. A second explanation for the plateau phenomenon could be that the intermediates produced by Escherichia intermedia are different in chemical nature from those produced by other strains. Such an explanation, although possible, is not probable, and it is not confirmed by the gas chromatographic analysis of metabolic products from Escherichia intermedia presented later in this report.

From Figures 49 and 50 it can be seen that there is an absence of metabolic intermediates when sorbitol is the substrate, as determined by comparison of COD and sorbitol COD curves.

The lack of intermediates accumulated when Escherichia intermedia was grown on sorbitol may point to the possibility that glucose and sorbitol are metabolized by completely different pathways. Bhatla and Gaudy have shown that the accumulation of intermediates when this organism is grown on glucose can be accounted for as volatile acids. However, the mechanism by which volatile acids are produced from glucose metabolism under aerobic conditions is not altogether clear. Strecker (113) has noted that Escherichia coli

can convert pyruvic acid to acetic and formic acids. In experiments with yeast, Maxon and Johnson (114) have shown that yeast converted glucose to ethanol when an excess of dissolved oxygen was present, and theorized that the oxidative enzymes of yeast could be saturated and the glycolytic enzymes which were present would account for the fermentation products detected. Based on the above observations, it could be suggested that the bacteria may produce acetic acid during growth due to a mechanism similar to that proposed by Maxon and Johnson. This would indicate that acetic acid intermediates would be produced only by facultative organisms; three of the identified organisms that have been used in the present work (Escherichia coli, Escherichia intermedia, and Serratia marcescens) are known to be facultative.

If the pathway for sorbitol metabolism is different from that of glucose, it may be such that it does not provide the opportunity for fermentative utilization resulting in volatile acid production. Horwitz and Kaplan (115) have isolated and purified from Bacillus subtilis the enzyme D-sorbitol dehydrogenase, which is capable of converting sorbitol to fructose, with additional enzymes subsequently converting fructose to xylitol and to xylulose, thereafter entering the glucuronate-gulonate pathway. Such a metabolic route might explain the lack of volatile acid intermediates when sorbitol is utilized.

Another possible mechanism to explain the lack of

metabolic intermediates in sorbitol metabolism could be that the first enzymatic reaction, which converts sorbitol to fructose is rate-limiting. In such a case, the fructose metabolism following the glycolytic pathway would not tend to utilize the fermentative route because the oxydative enzymes would not be saturated with reduced substrate.

It is interesting to note that a considerable portion of the COD in the medium can still be present after the log growth phase has ended. Figure 51 illustrates such a case with Escherichia intermedia in the presence of glucose. In this case, approximately 68 per cent of the COD applied was present after the growth curve no longer fitted a straight line when plotted on semi-log paper. The same relationship was observed in most cases studied, with 50 per cent or more of the applied COD present at the end of the log growth phase.

8. Sequential Substrate Removal

In experiments to examine the possibility of sequential substrate removal for the test organisms, biological solids concentration, COD removal, glucose COD, and sorbitol COD curves were utilized to study the biochemical growth response of various pure cultures acclimated to sorbitol and grown in a glucose-sorbitol medium. Figure 52 shows the results obtained from an experiment using Blue organism. The COD uptake and biological solids curves were similar to those usually observed for a culture incubated with a single carbon source. However, analysis of the substrate removal

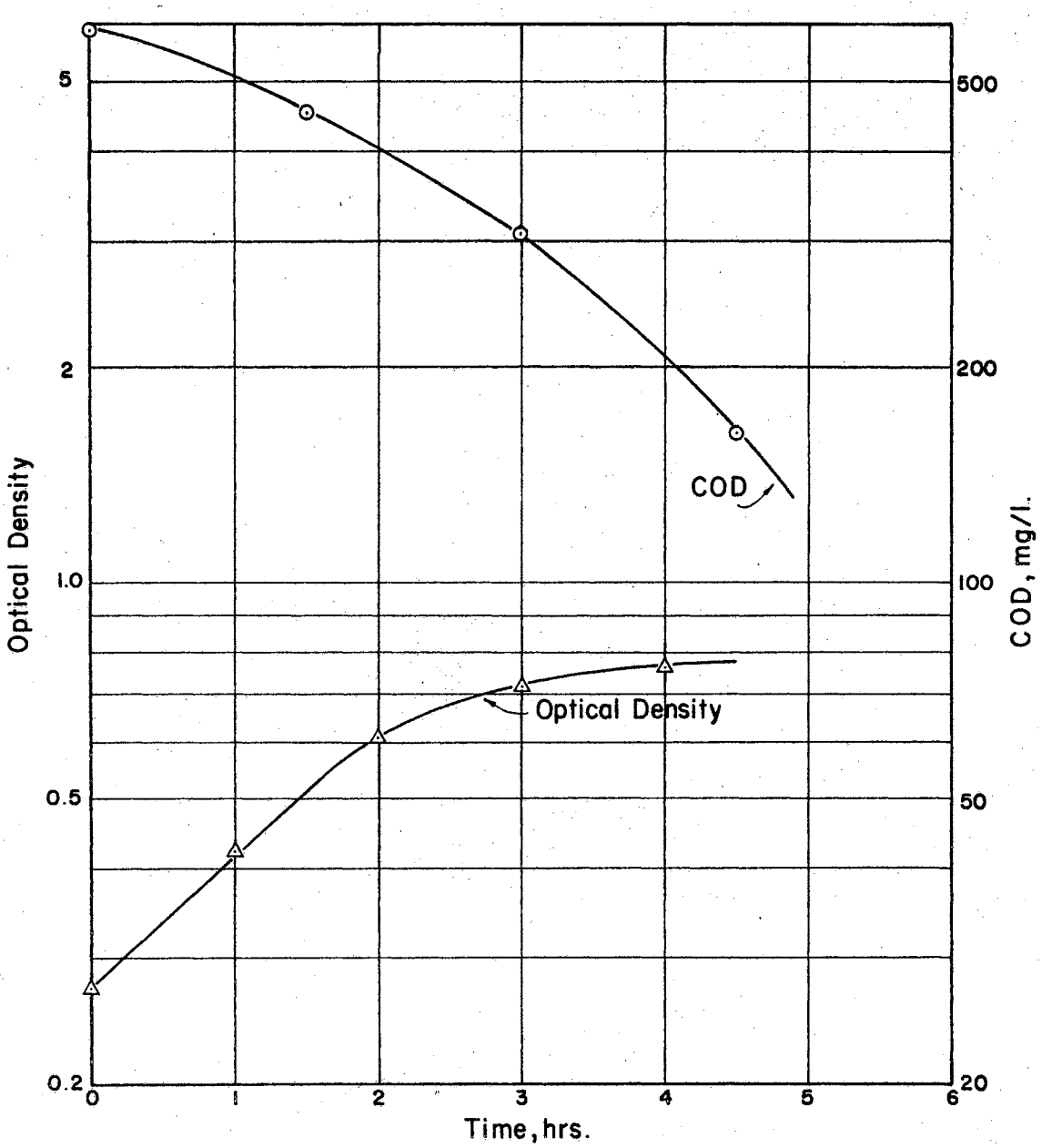

Figure 51. Relationship of COD removal and growth rate for Escherichia intermedia.

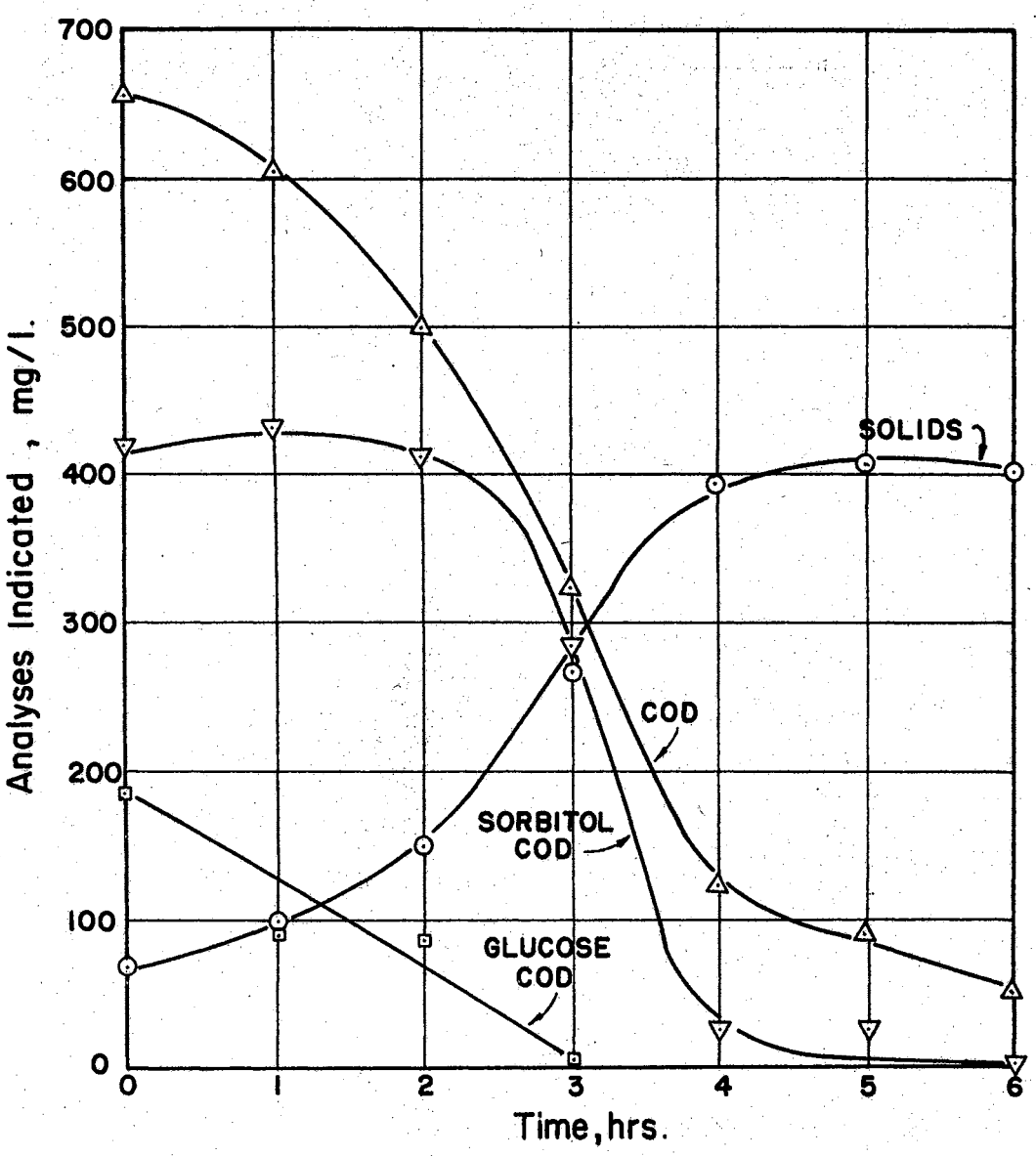

Figure 52. System behavior of Blue organism acclimated to sorbitol.

curve indicated that sorbitol was not metabolized to a significant extent until the glucose had been removed from the medium.

In Figure 53 results of a similar experiment performed with Escherichia intermedia are shown. The diphasic growth characteristic of the organism when using glucose was not observed when grown on glucose and sorbitol in combination. Examination of the substrate removal curves shows that sorbitol remained in the medium until the glucose was removed. In the same manner, Figure 54 indicates that Serratia marcescens metabolized glucose before using sorbitol.

Analysis

Examination of the data collected reveals that with the three organisms studied, glucose was removed before sorbitol. Metabolism of sorbitol is shown in these experiments (Figures 52, 53, and 54) to take place immediately after glucose exhaustion, without evidence for a diphasic curve for either COD utilization or biological solids concentration. Relating these observations to the previous glucose-sorbitol medium experiments reported in the growth rate comparison experiments, it can be surmised that sequential substrate removal did take place with the experiments using sorbitol-acclimated seed shown in Figures 28, 29, and 30. Acclimation to sorbitol, however, did prevent the effect from being observed as a growth plateau in the two-substrate systems shown in Figures 28-30. It is obvious that if the same experiments reported in this

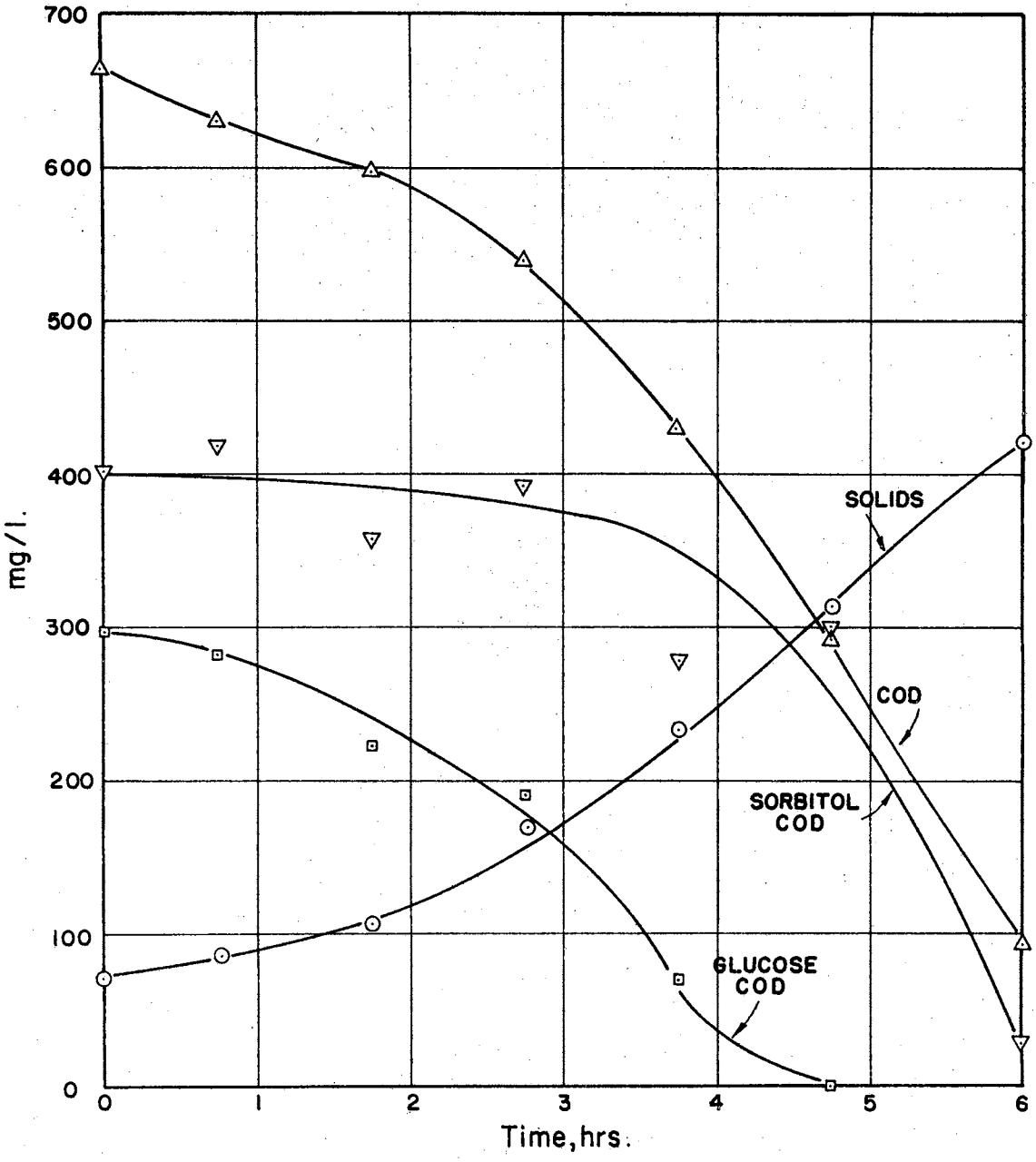

Figure 53. System behavior of Escherichia intermedia acclimated to sorbitol.

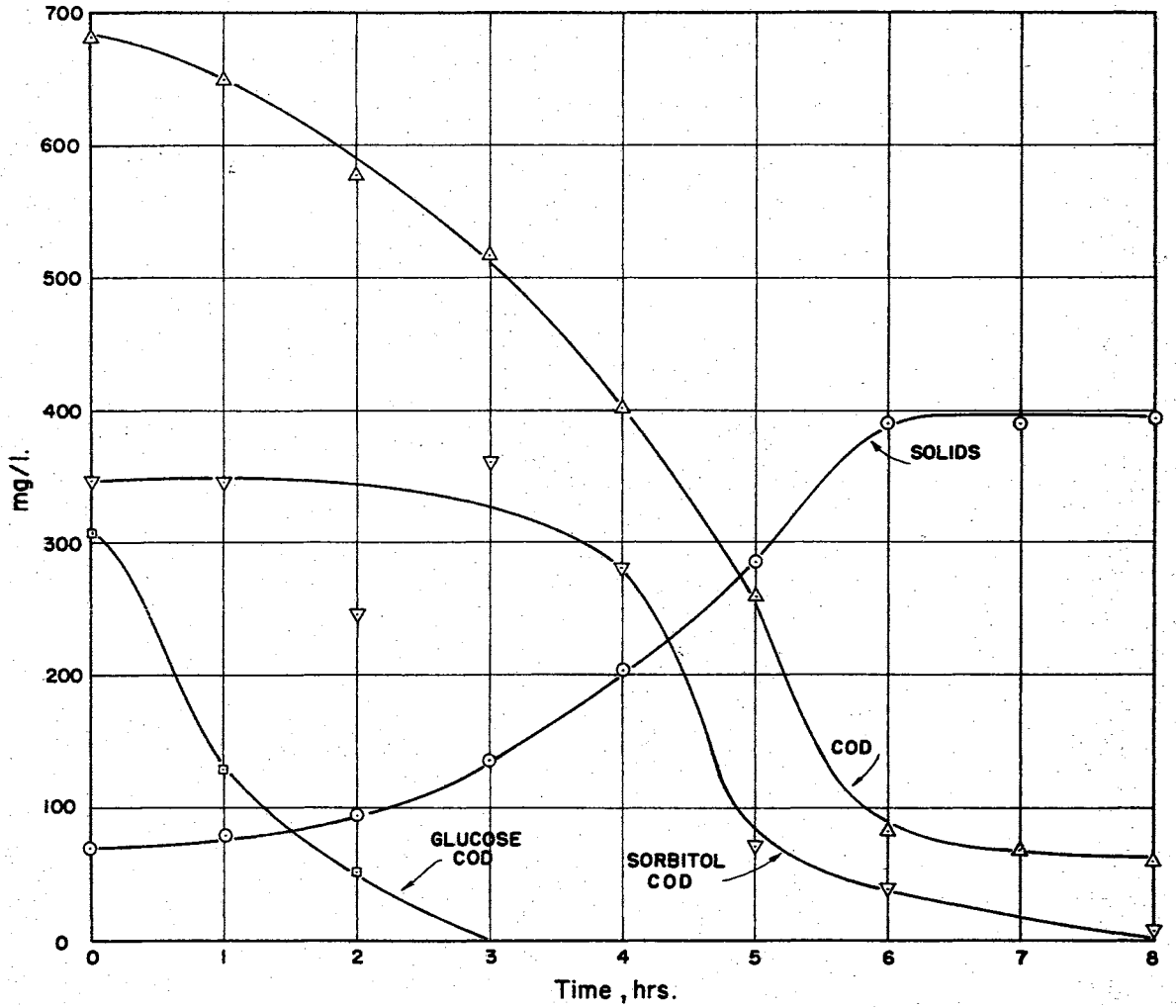

Figure 54. System behavior of Serratia marcescens acclimated to sorbitol.

section were to be performed using a seed acclimated to glucose, the same basic biochemical response would have been registered, but most likely with a period of acclimation noted between the two phases of active substrate uptake. These experiments provide excellent confirmation of the work of Gaudy, Komolrit, and Bhatla (109), and Gaudy, Gaudy, and Komolrit (110), who first studied the causes and effects of sequential substrate removal and proposed the mechanisms of the suppression of an existing enzyme system. These experiments also attest further to the fact that sequential utilization of glucose and sorbitol is of fairly general occurrence.

Gaudy, Gaudy, and Komolrit (110) have reported on sequential substrate removal by a pure culture of Escherichia coli using a glucose-sorbitol medium. Since enzyme repression now appears to be common for a variety of organisms growing on the substrates herein employed, the phenomenon of sequential substrate removal may be expected to affect all or most of the species in a system. Therefore, it seems likely that little or no predominance change would be expected from the effect of this mechanism in a mixed culture, unless one of the carbon sources could not be utilized by one or more of the species present.

C. Batch Studies with Mixed Cultures

1. Mixed Cultures--Two Organisms on Glucose

The experiments in this section were performed by setting up three different systems at the same substrate

concentration: one for each pure culture and one for the mixed system. Viable count determinations and oxygen uptake measurements were made for all systems, as well as COD removal, glucose COD utilization, and biological solids production. The biochemical behavior of the pure cultures was not investigated, since the properties of these systems had been studied earlier. Approximately 500 mg/l glucose were used as a carbon source in these experiments.

The results have been organized for presentation in five different sections, as follows: a. mixtures of two fast-growing organisms; b. mixtures of a fast-growing and a medium fast-growing organism; c. mixtures of a fast-growing and a slow-growing organism; d. mixtures of two medium fast-growing organisms; e. mixtures of a medium fast-growing and a slow-growing organism.

a. Experiments Including Mixtures of Two Fast-growing Organisms

Figure 55 shows viable count determinations of the pure cultures and the mixed system for an experiment using Pseudomonas aeruginosa and Escherichia intermedia. From this figure it can be seen that the growth patterns of the pure cultures and of each individual culture in the mixed system were similar. Escherichia intermedia in pure culture showed its typical secondary growth after a plateau, but such was not observed in the mixed culture. Figure 56 shows the biochemical behavior of the mixed system. It is noted that no secondary oxygen uptake took place in the

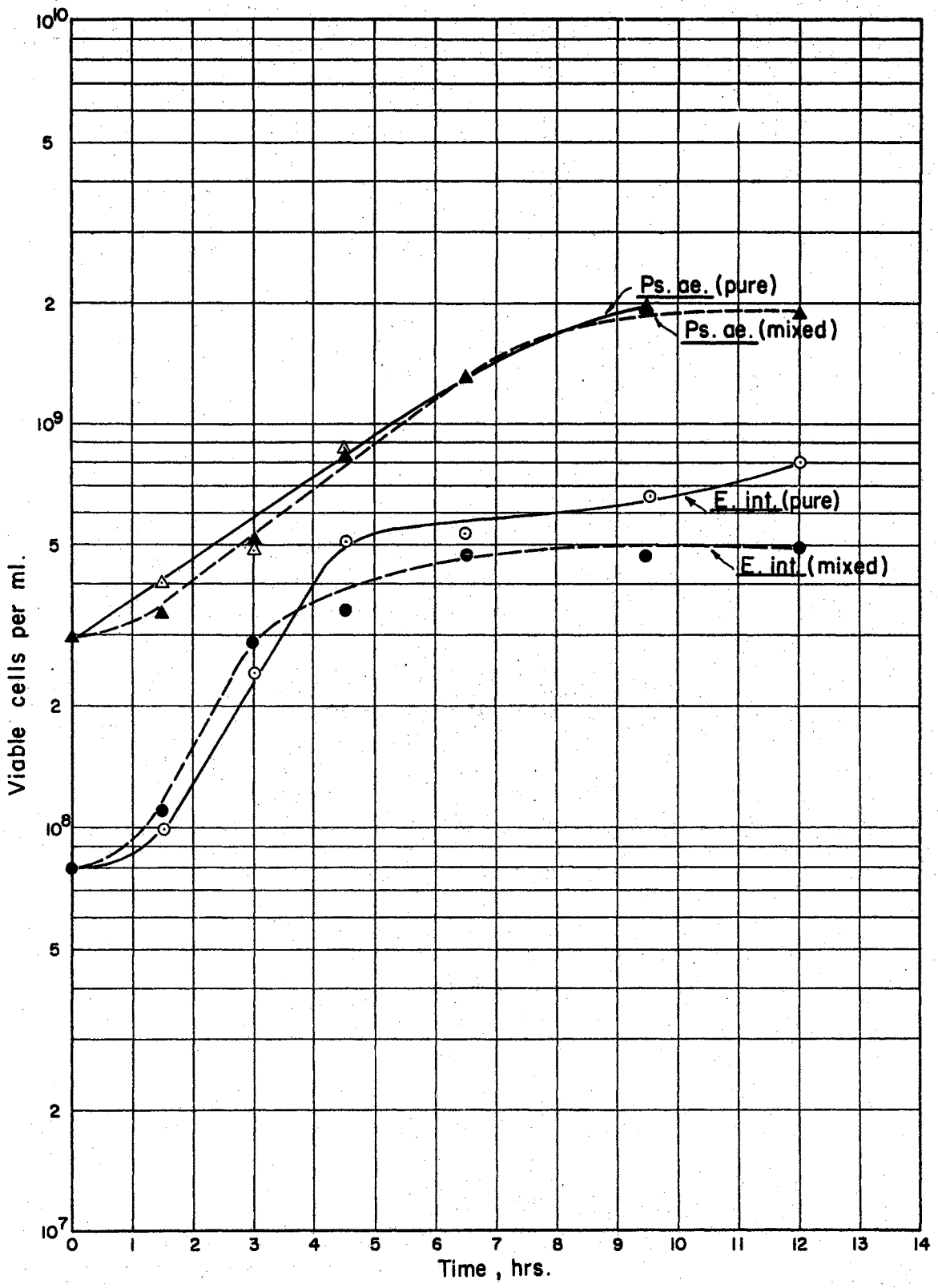

Figure 55. Viable count for Pseudomonas aeruginosa and Escherichia intermedia in a mixed system.

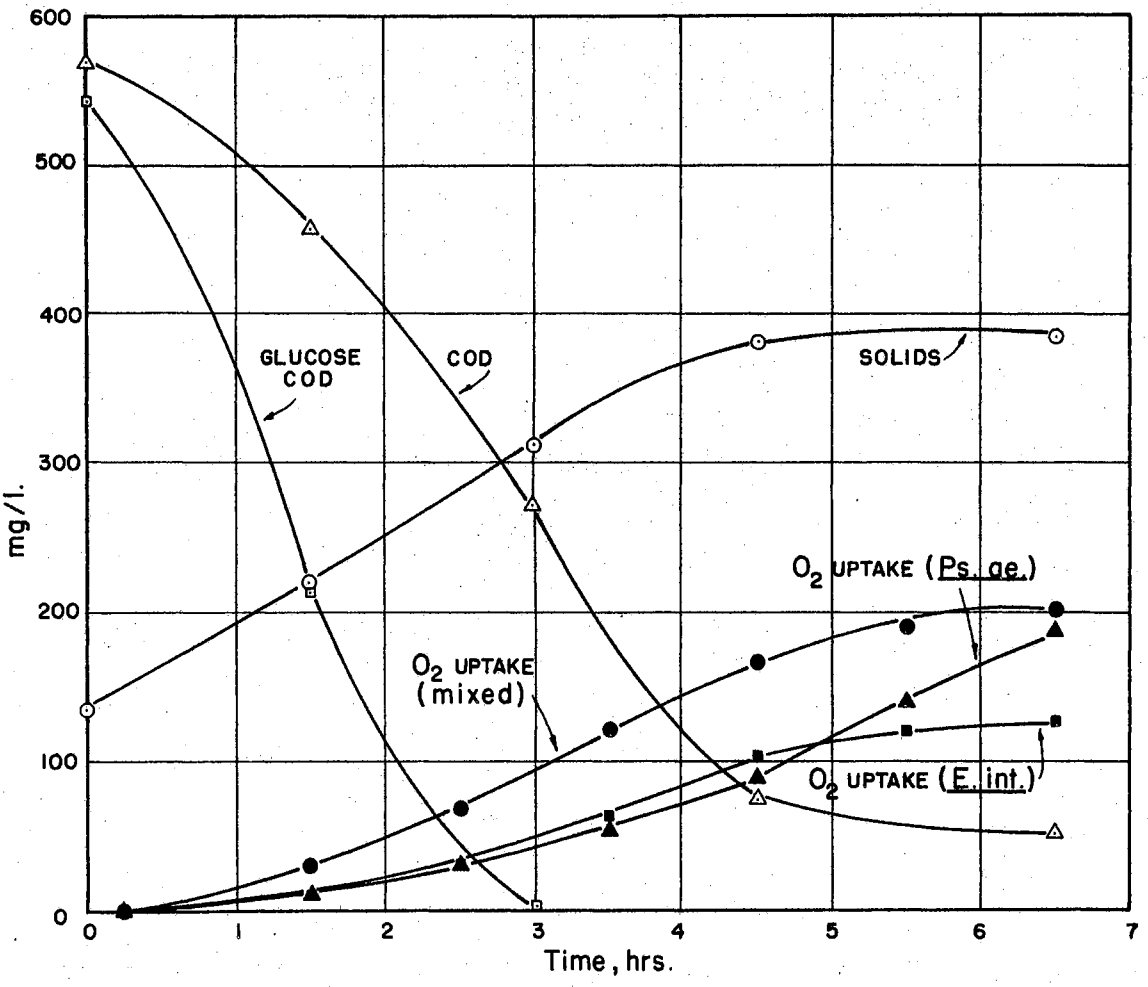

Figure 56. Biochemical response of a mixed system of Pseudomonas aeruginosa and Escherichia intermedia.

case of the mixed culture, although the plateau in oxygen uptake was quite evident for the pure culture. COD and glucose were removed very rapidly in the mixed system. An examination of the oxygen uptake curve shows that during the period of glucose removal, oxygen uptake in the combined system was approximately equal to the sum of the oxygen uptake for the individual organisms.

Results of an experiment using the two fast-growing cultures, Blue organism and Pseudomonas aeruginosa are shown in Figures 57 and 58. The viable counts show that there was little difference between the mixed and pure culture growth curves, although the mixed curves end with somewhat lower numbers of viable cells. Figure 58 indicates a very rapid uptake of COD and glucose with a correspondingly rapid increase in biological solids. Inspection of the oxygen uptake curves shows a slightly more rapid uptake for the mixed system (for 2 1/4 hours) than the sum of the pure culture systems. The cell yields obtained from these and all other experiments in this group are given in Table V.

b. Experiments with Mixtures of Fast and Medium-fast Growing Organisms

In this group of experiments Serratia marcescens was combined with Blue organism (Figures 59 and 60), with Escherichia intermedia (Figures 61 and 62), and with Pseudomonas aeruginosa (Figures 63 and 64). In all of these experiments the mixtures responded in a very similar manner.

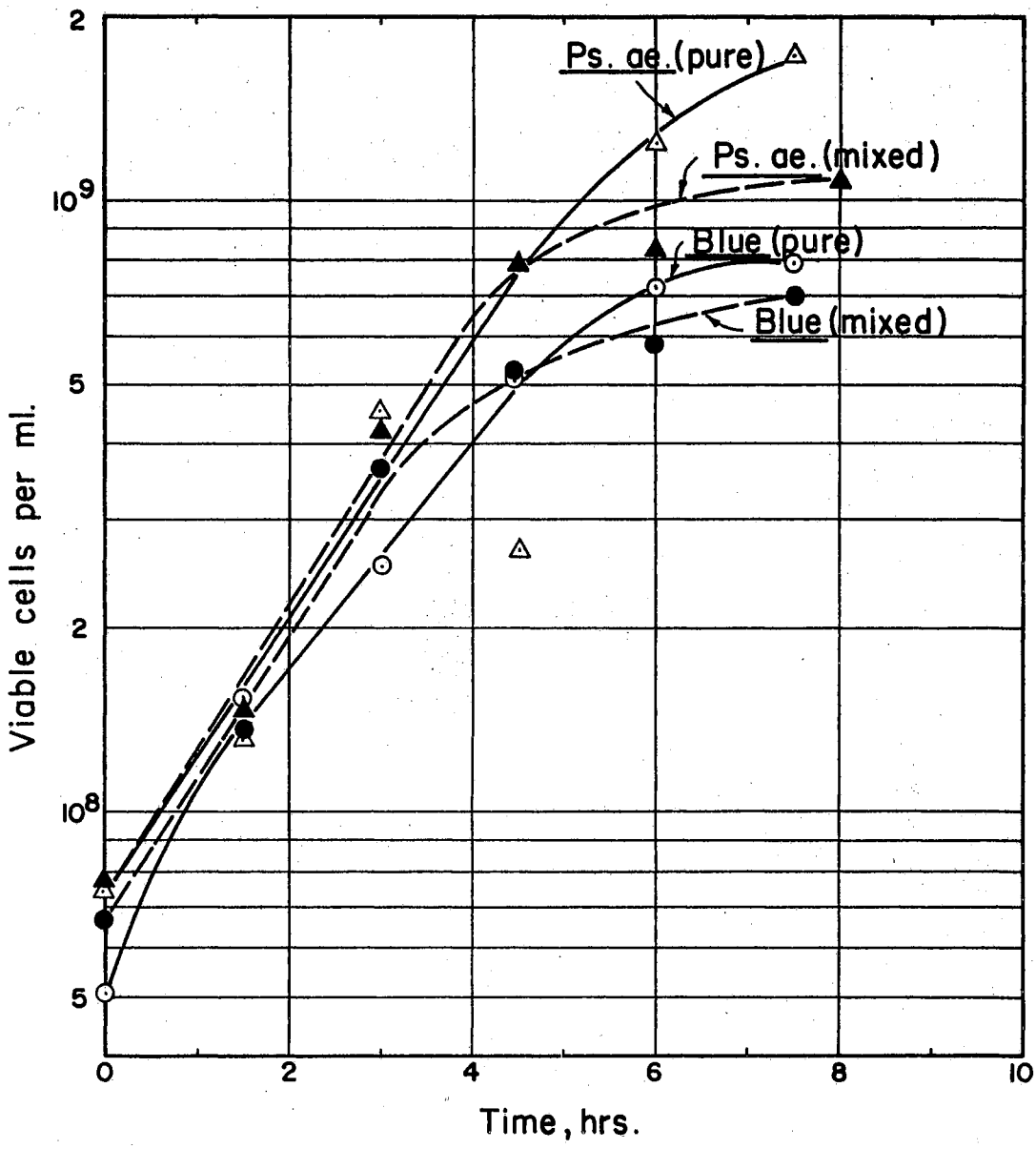

Figure 57. Viable count for Pseudomonas aeruginosa and Blue organism in a mixed system.

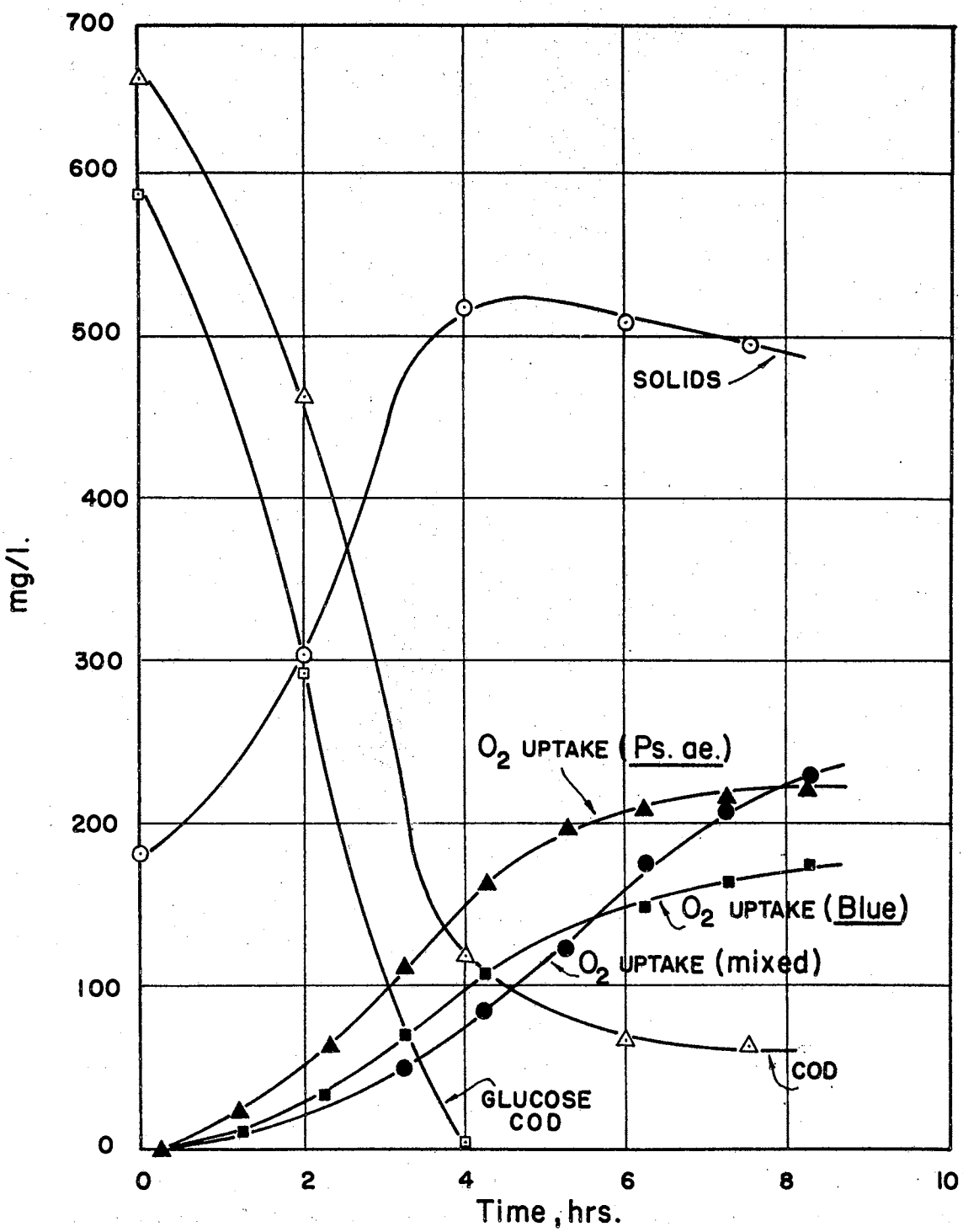

Figure 58. Biochemical response of a mixed system of Pseudomonas aeruginosa and Blue organism.

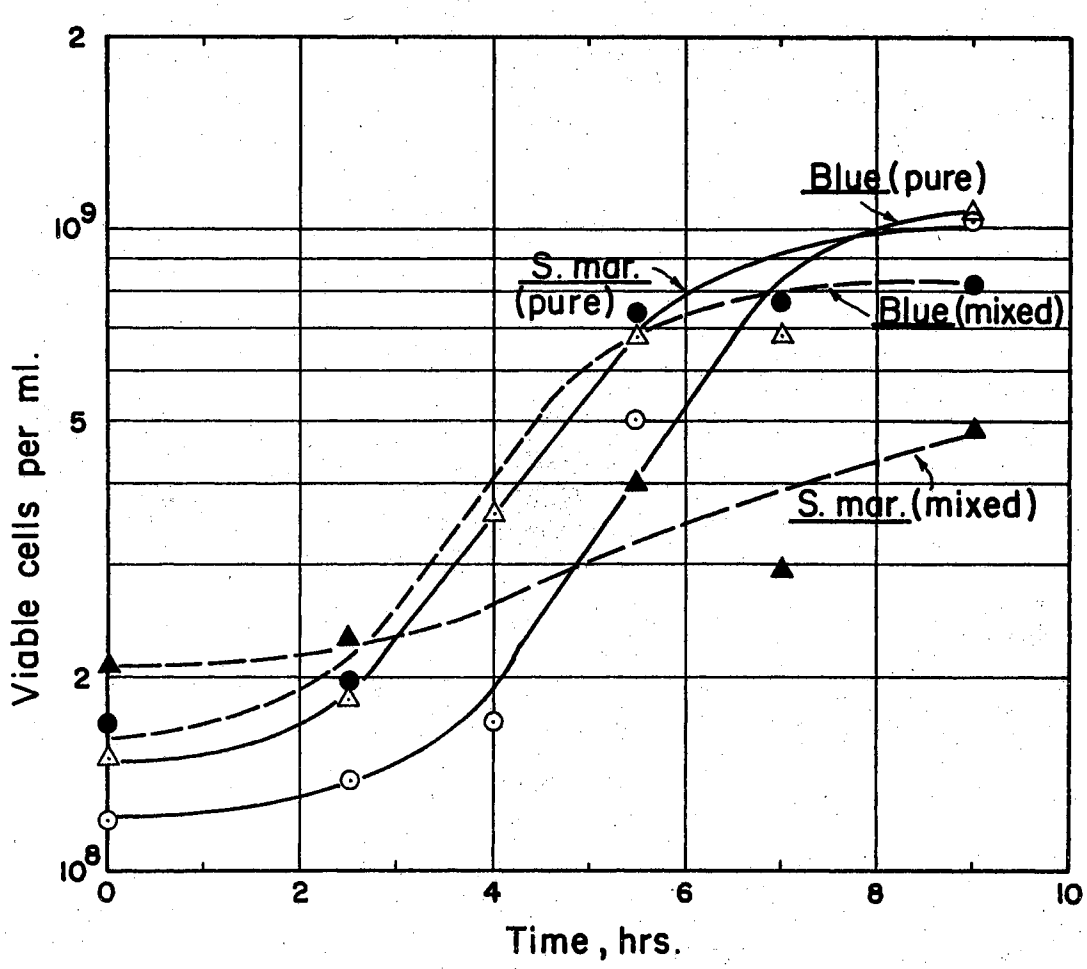

Figure 59. Viable count for Blue organism and Serratia marcescens in a mixed system.

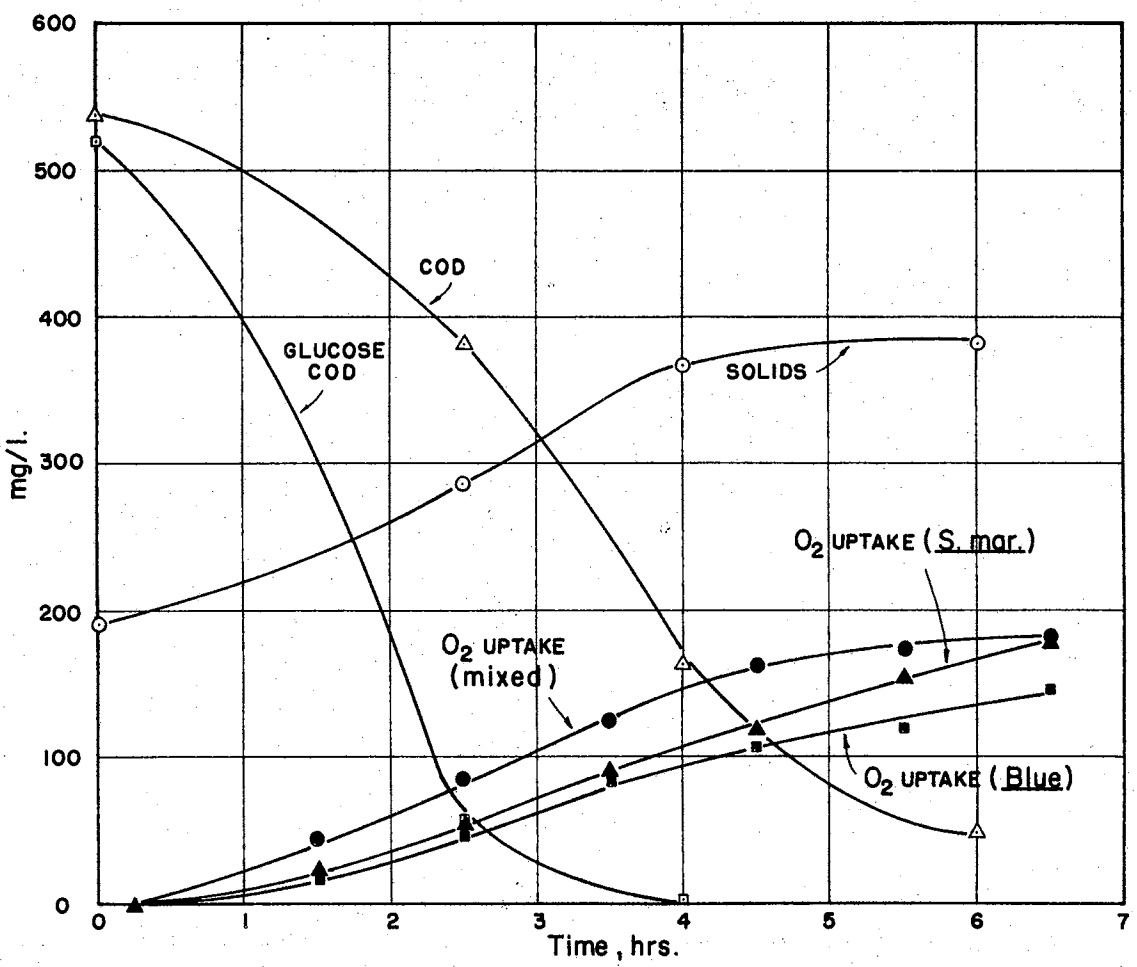

Figure 60. Biochemical response of a mixed system of Blue organism and Serratia marcescens.

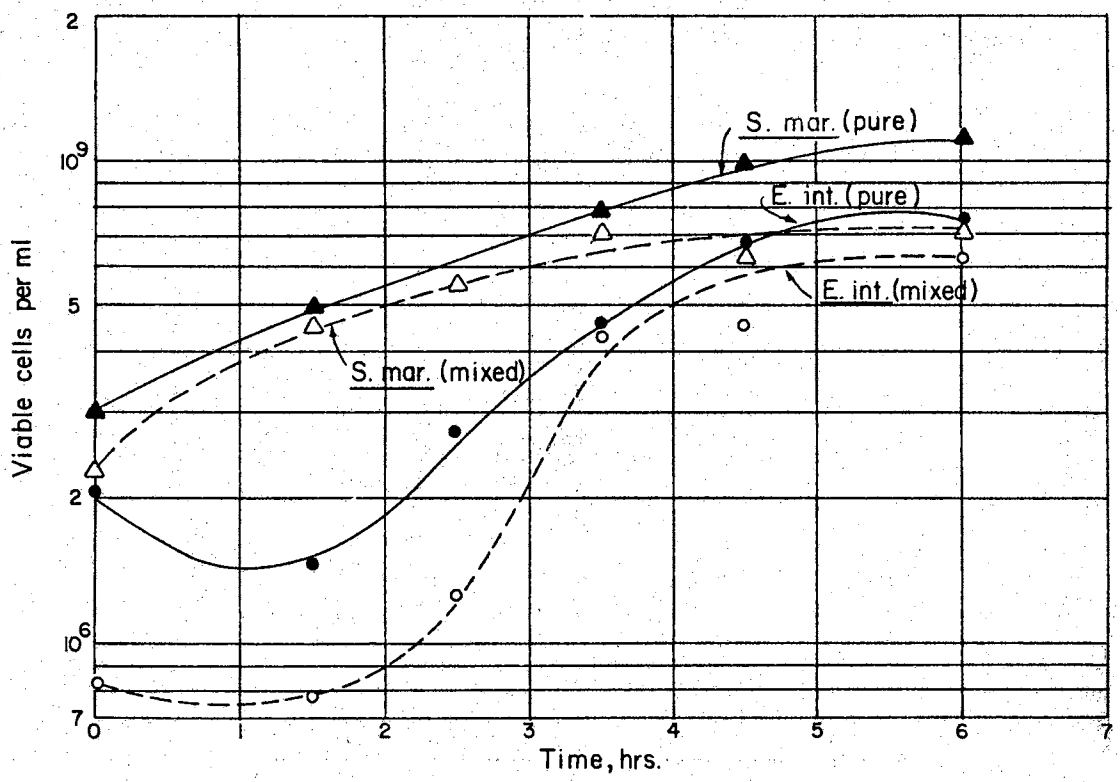

Figure 61. Viable count for Escherichia intermedia and Serratia marcescens in a mixed system.

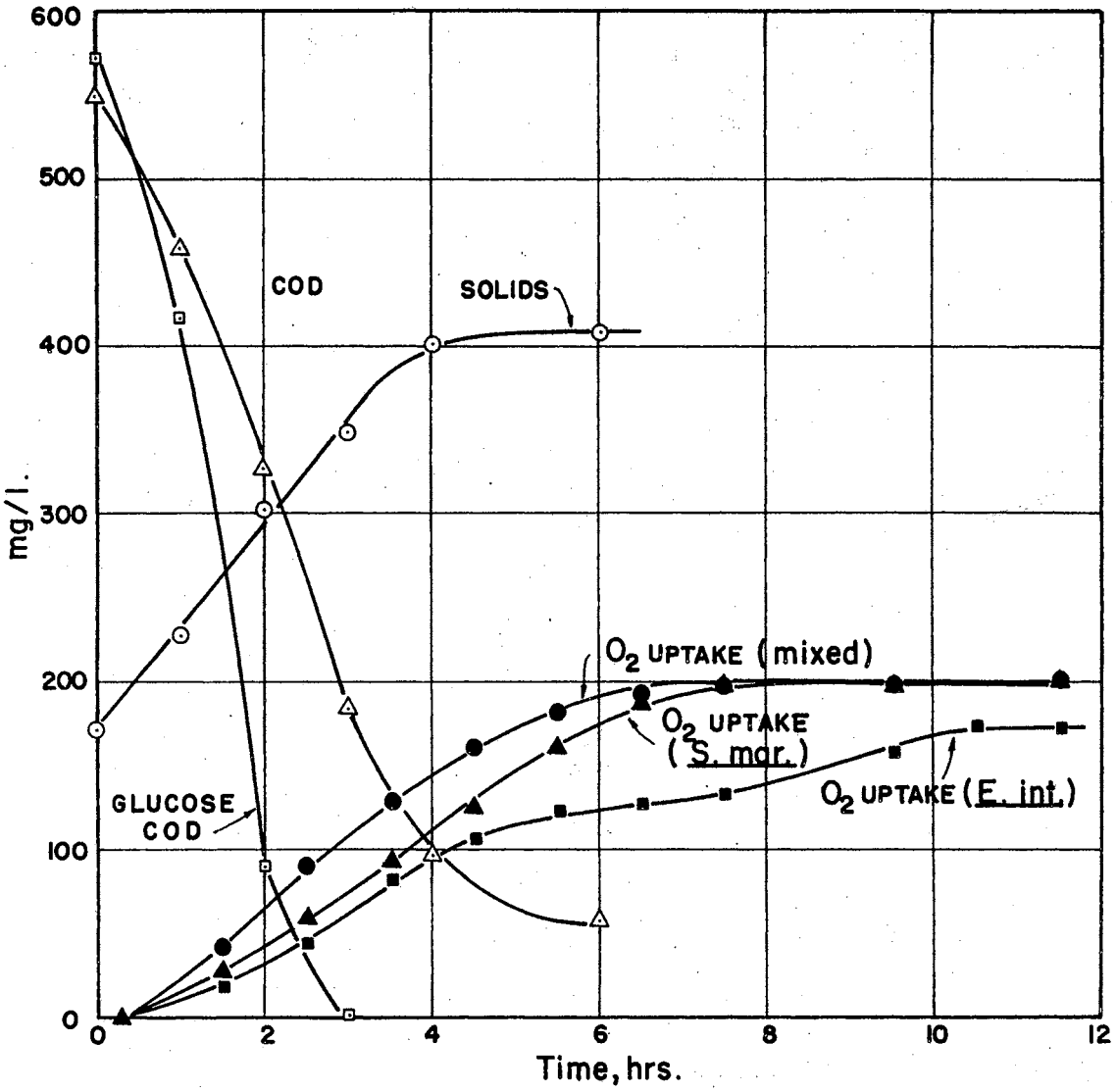

Figure 62. Biochemical response of a mixed system of Escherichia intermedia and Serratia marcescens.

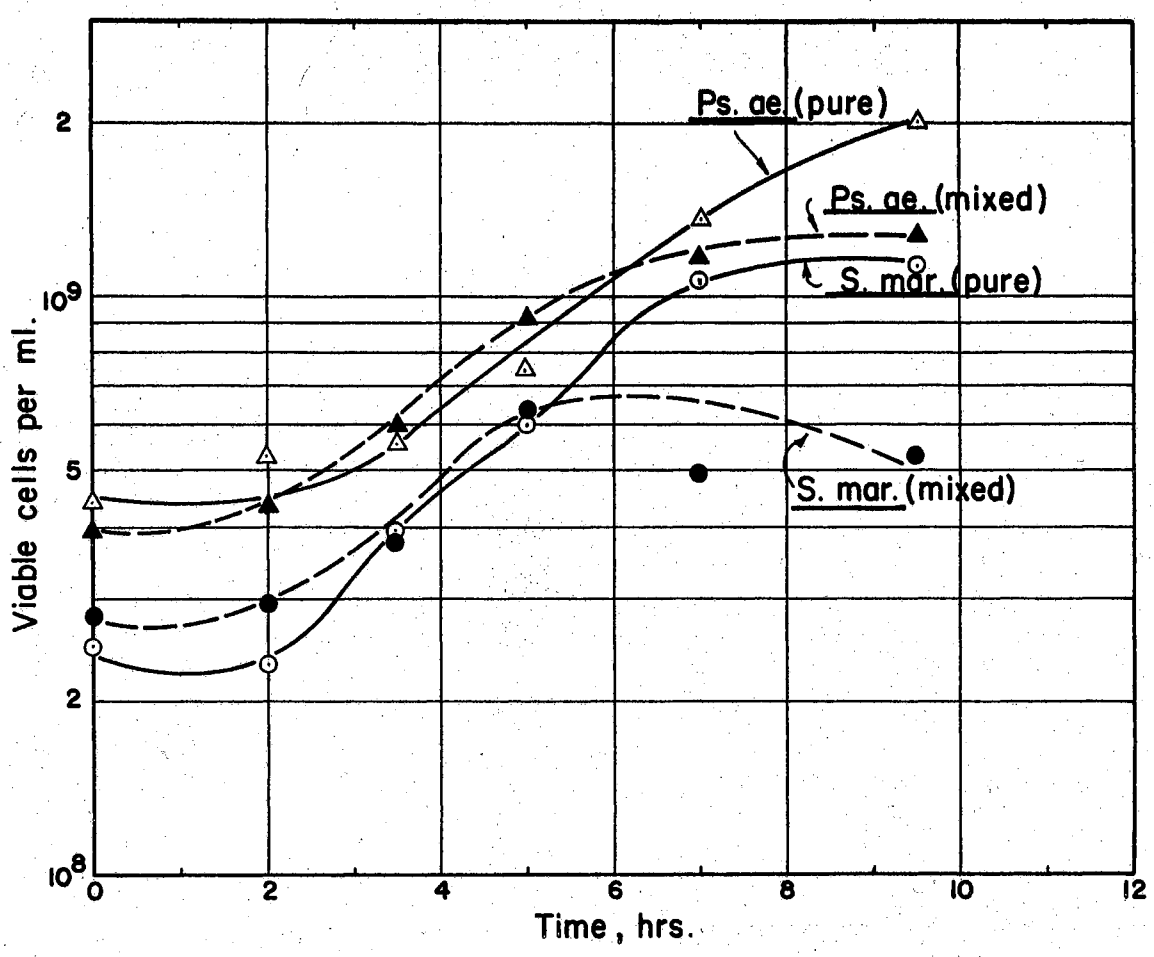

Figure 63. Viable count for Pseudomonas aeruginosa and Serratia marcescens in a mixed system.

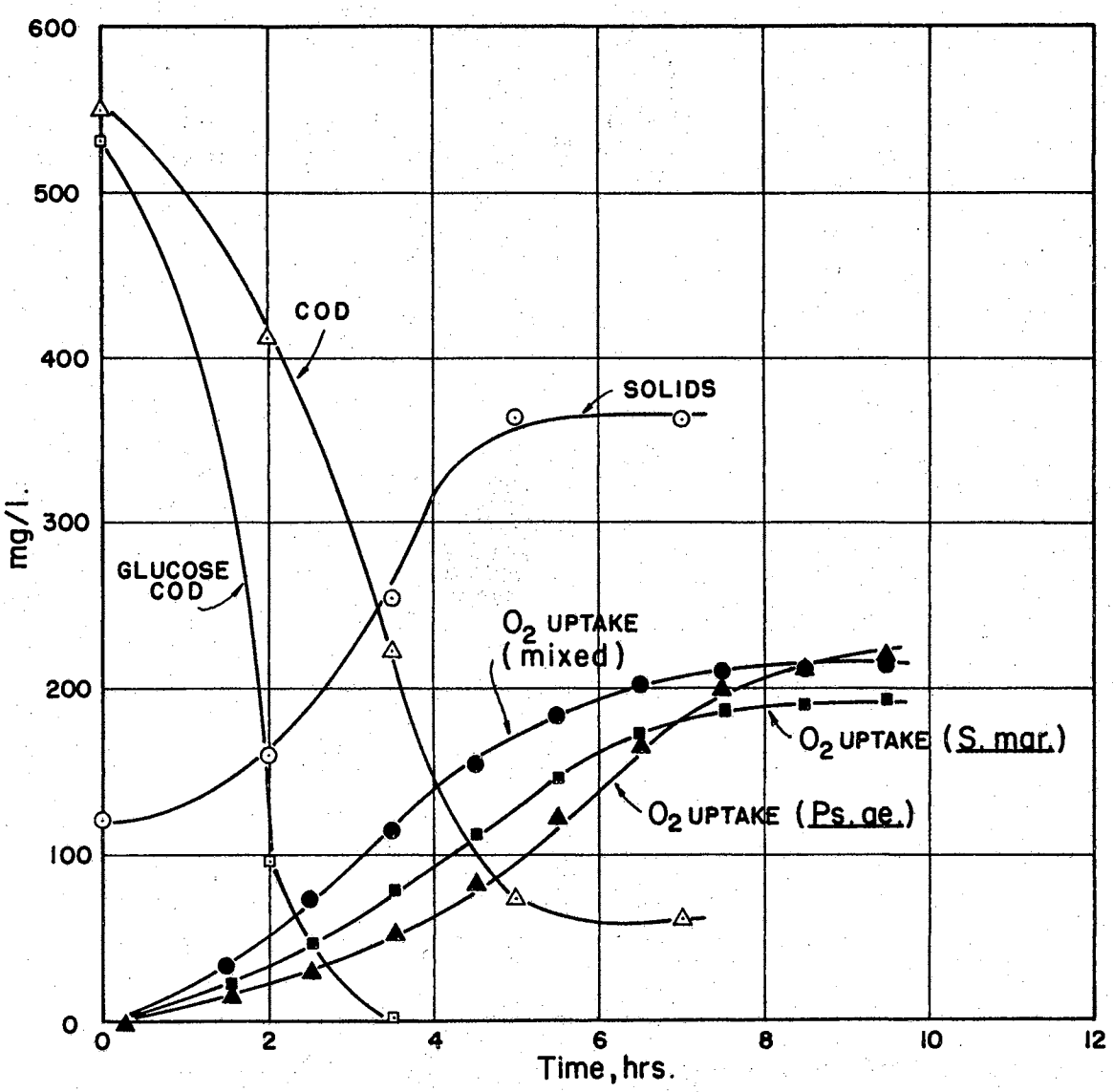

Figure 64. Biochemical response of a mixed system of Pseudomonas aeruginosa and Serratia marcescens.

There was rapid glucose and COD removal with production of metabolic intermediates and/or end products. The viable count growth curves (Figures 59, 61, and 63) show that in all three cases the numbers of each of the organisms in the mixed systems were lower than their respective numbers in the pure culture controls, with the depressing effect being most marked in the case of Serratia marcescens. From Figure 62 it may be noted that the oxygen uptake plateau was not present with the mixed culture system, although it could be detected from the Escherichia intermedia pure culture data.

Figures 65 and 66 show the results of an experiment in which Escherichia intermedia was combined with Yellow organism. From Figure 66 it can be seen that no oxygen uptake plateau was observed in the mixed system. An increase in the activity of the mixed system may be noted from the oxygen uptake curves. From Figure 65 it may be seen that after a period of growth, the viable population of Yellow organism died off more rapidly than did Escherichia intermedia.

In Figures 67 and 68 results of an experiment including Blue organism and Yellow organism are shown. The viable cell counts (Figure 67) show that Yellow organism grew very little when incubated in a mixed culture with Blue organism.

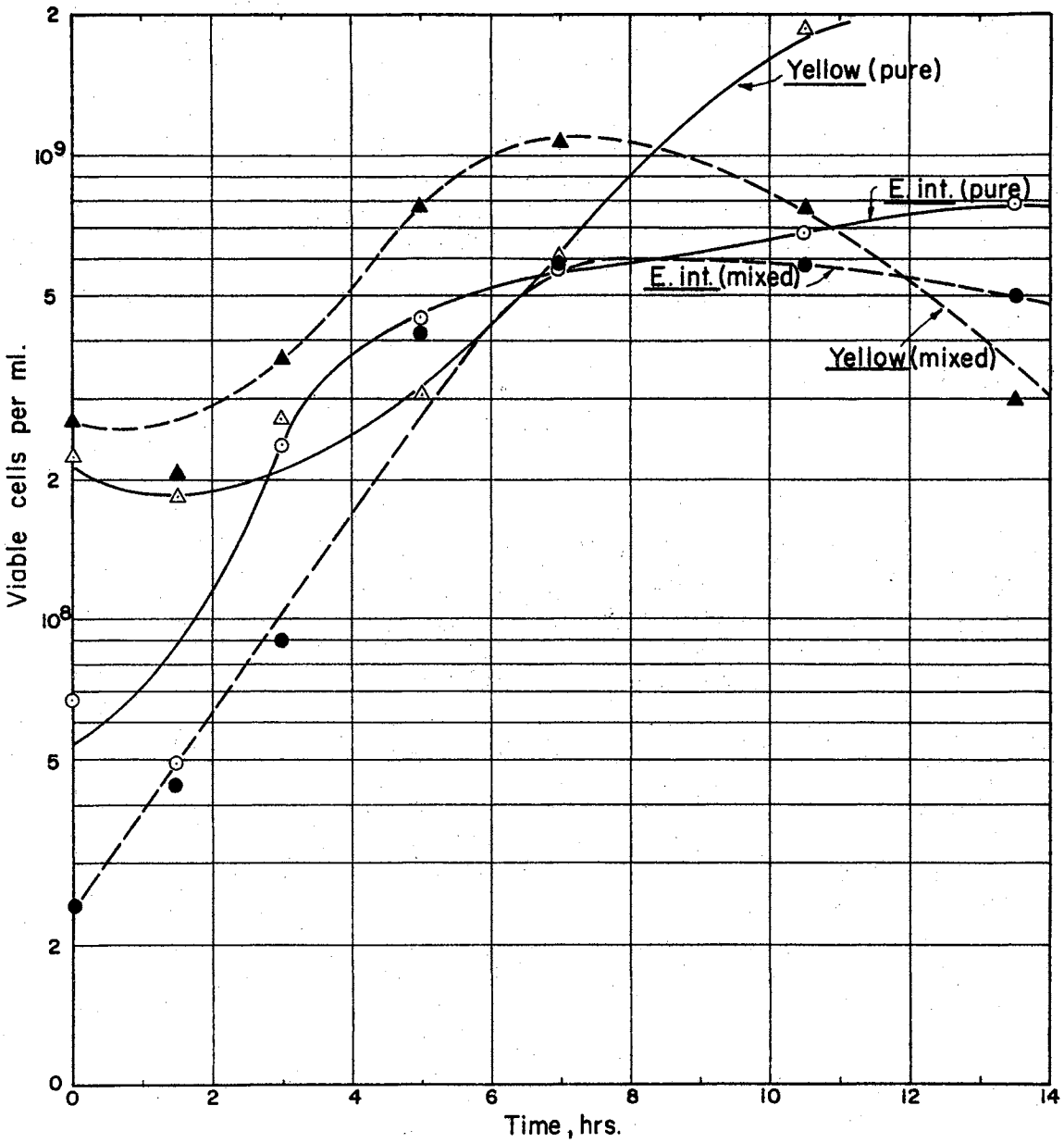

Figure 65. Viable count for Escherichia intermedia and Yellow organism.

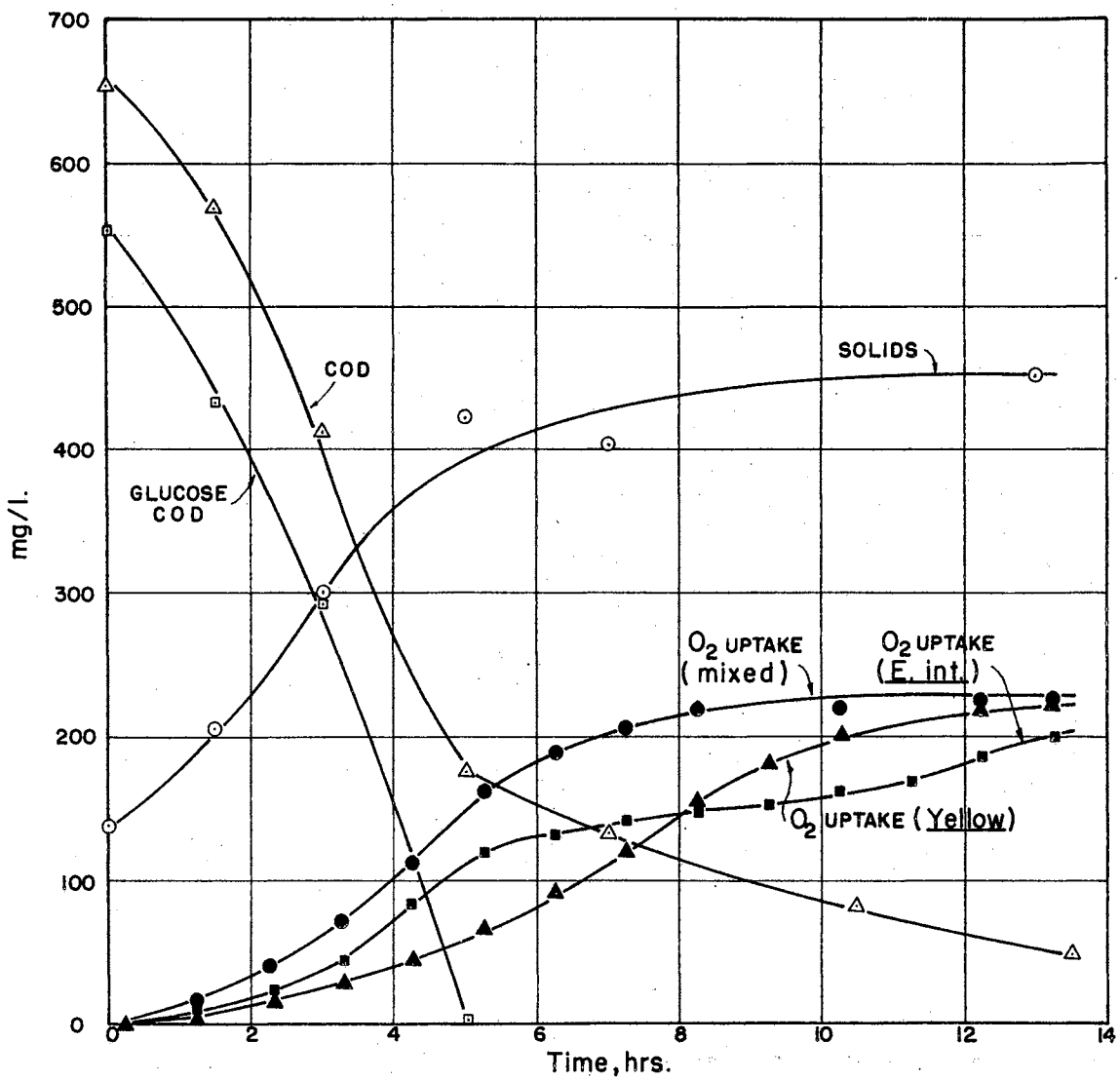

Figure 66. Biochemical response of a mixed system of Escherichia intermedia and Yellow organism.

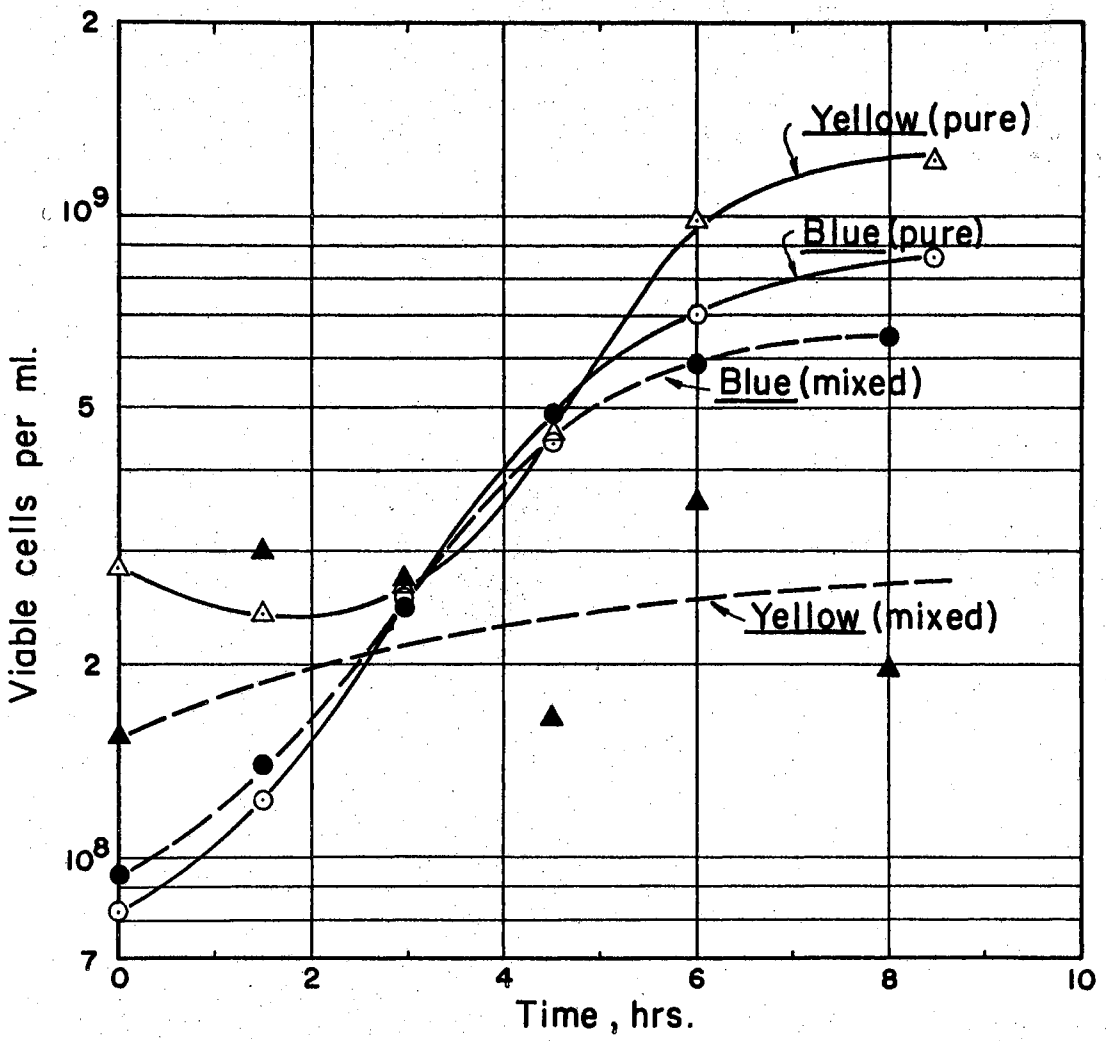

Figure 67. Viable count for Blue organism and Yellow organism in a mixed system.

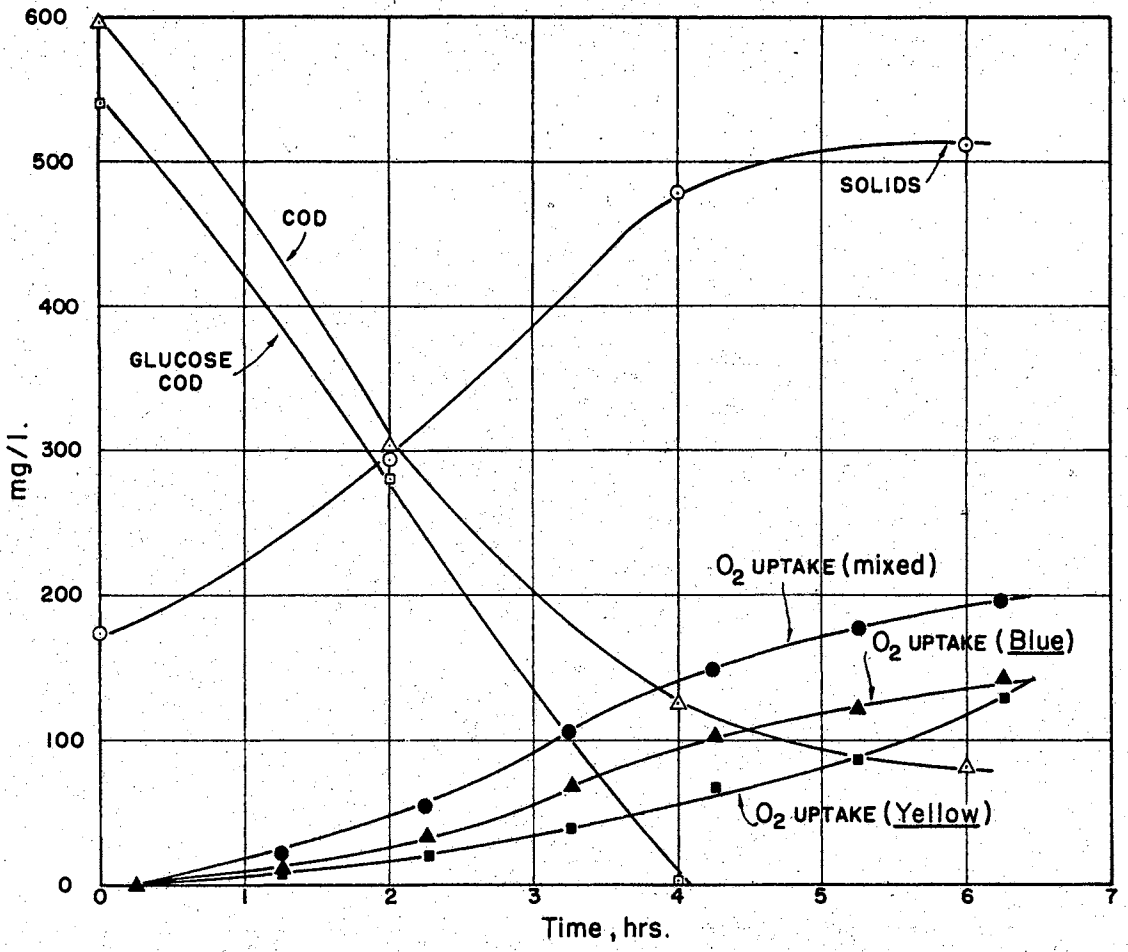

Figure 68. Biochemical response of a mixed system of Blue organism and Yellow organism.

c. Experiments Employing Mixtures of Fast-growing and Slow-growing Organisms

A mixture of Pseudomonas aeruginosa and Escherichia coli was used with the results shown in Figures 69 and 70. It may be seen that the oxygen uptake of the mixed system was larger than the sum of the uptakes of the pure cultures up to 5 1/2 hours (Figure 70). From the viable counts (Figure 69) it may be seen that although no effect was observed on the growth of Escherichia coli in mixed culture, its lag phase appeared to be reduced.

d. Experiments Including Mixtures of Two Medium-fast Growing Organisms

Results of the experiments in which Serratia marcescens was combined with Yellow organism are shown in Figures 71 and 72. The viable count data as well as the oxygen uptake data show that the Yellow organism did not grow to any appreciable extent in the mixed system.

e. Experiments Employing Mixtures of Medium-fast Growing Organisms and Slow-growing Organisms

Results of an experiment with Serratia marcescens and Escherichia coli are shown in Figures 73 and 74. Growth of both organisms in the mixed system in terms of viable count was somewhat depressed in relation to the pure culture controls.

Results of an experiment with Yellow organism and Escherichia coli are shown in Figures 75 and 76. An increase in activity was noted in the mixed system, as

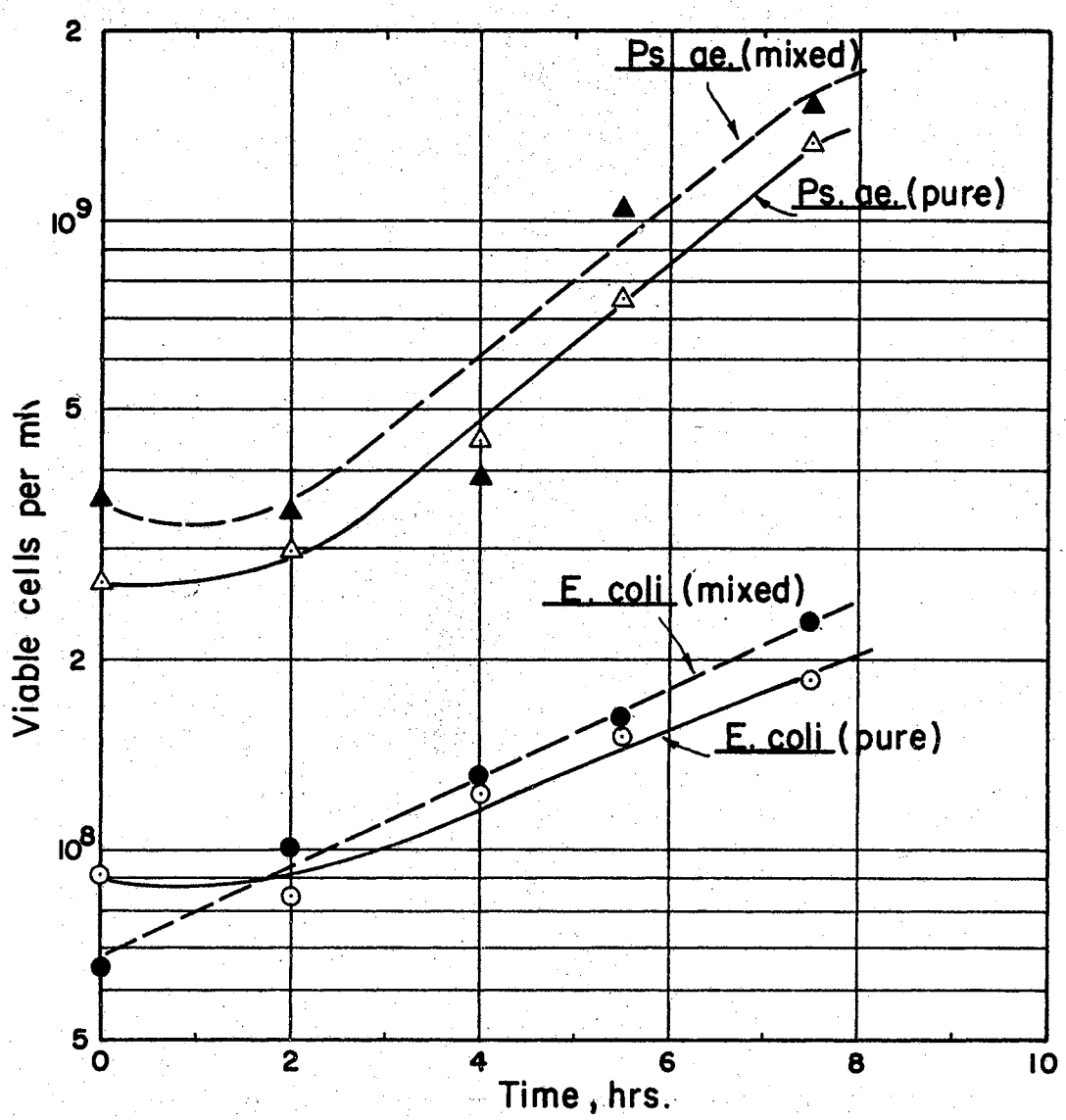

Figure 69. Viable count for Pseudomonas aeruginosa and Escherichia coli K-12.

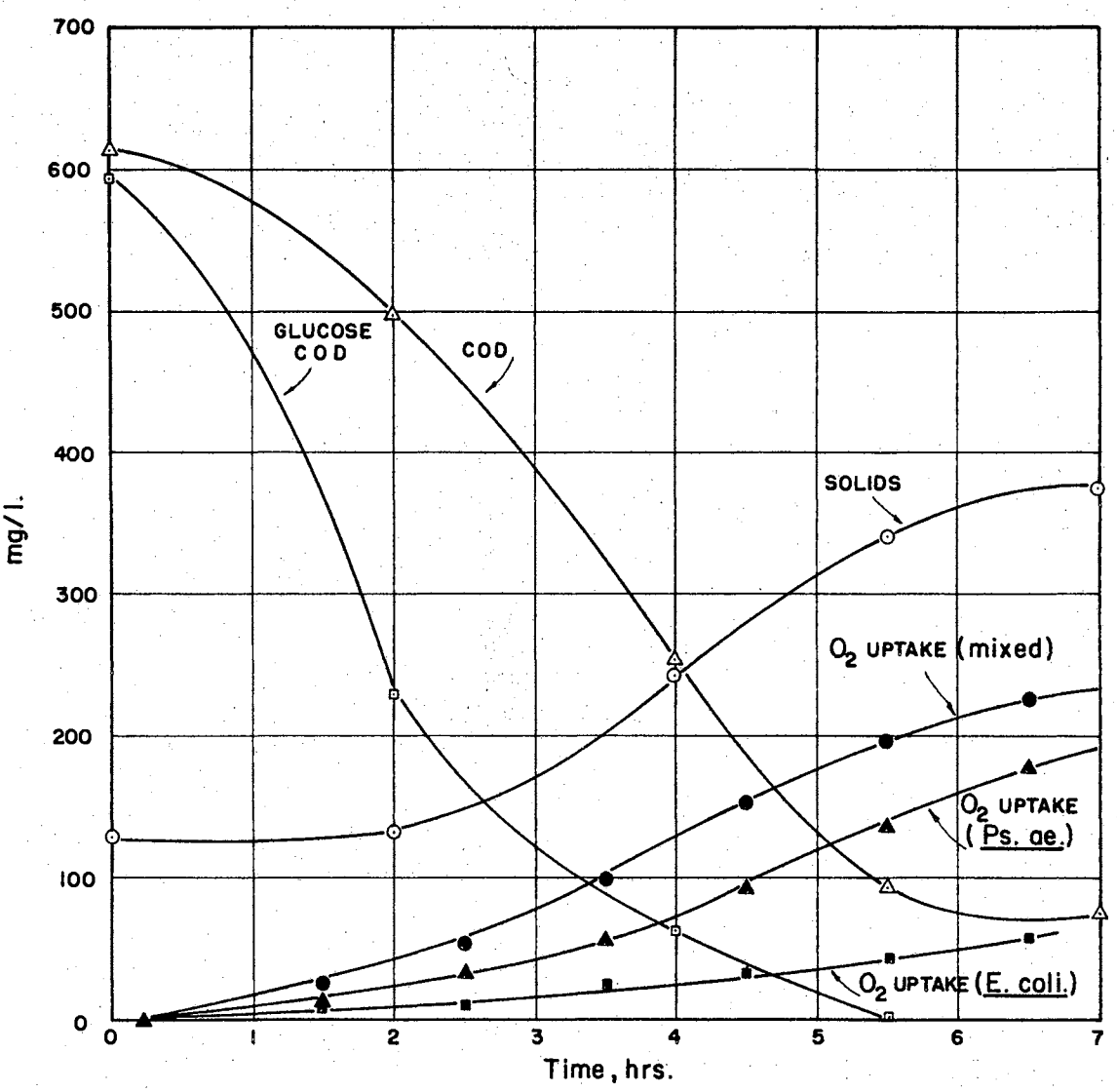

Figure 70. Biochemical response of a mixed system of Pseudomonas aeruginosa and Escherichia coli.

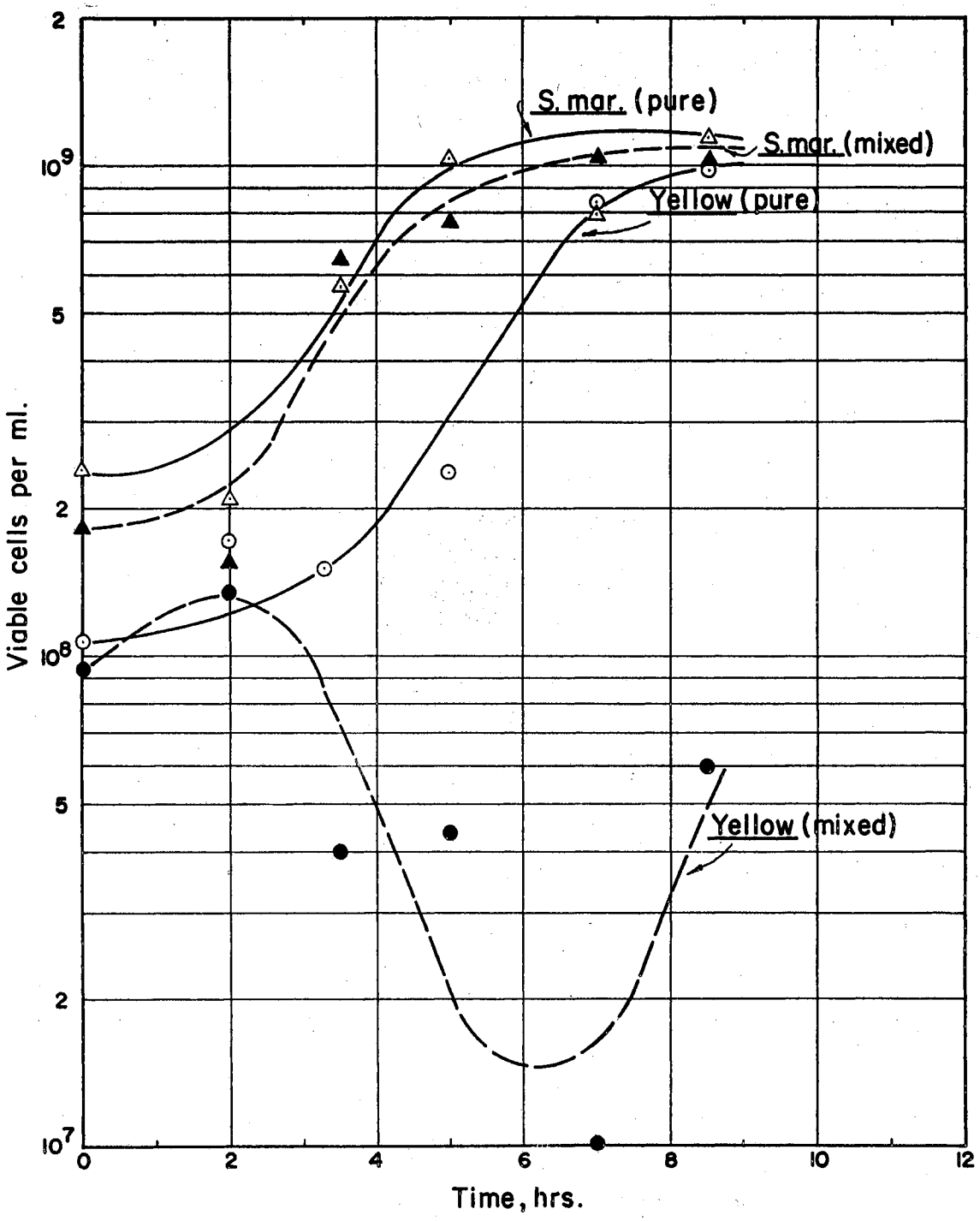

Figure 71. Viable count for Serratia marcescens and Yellow organism.

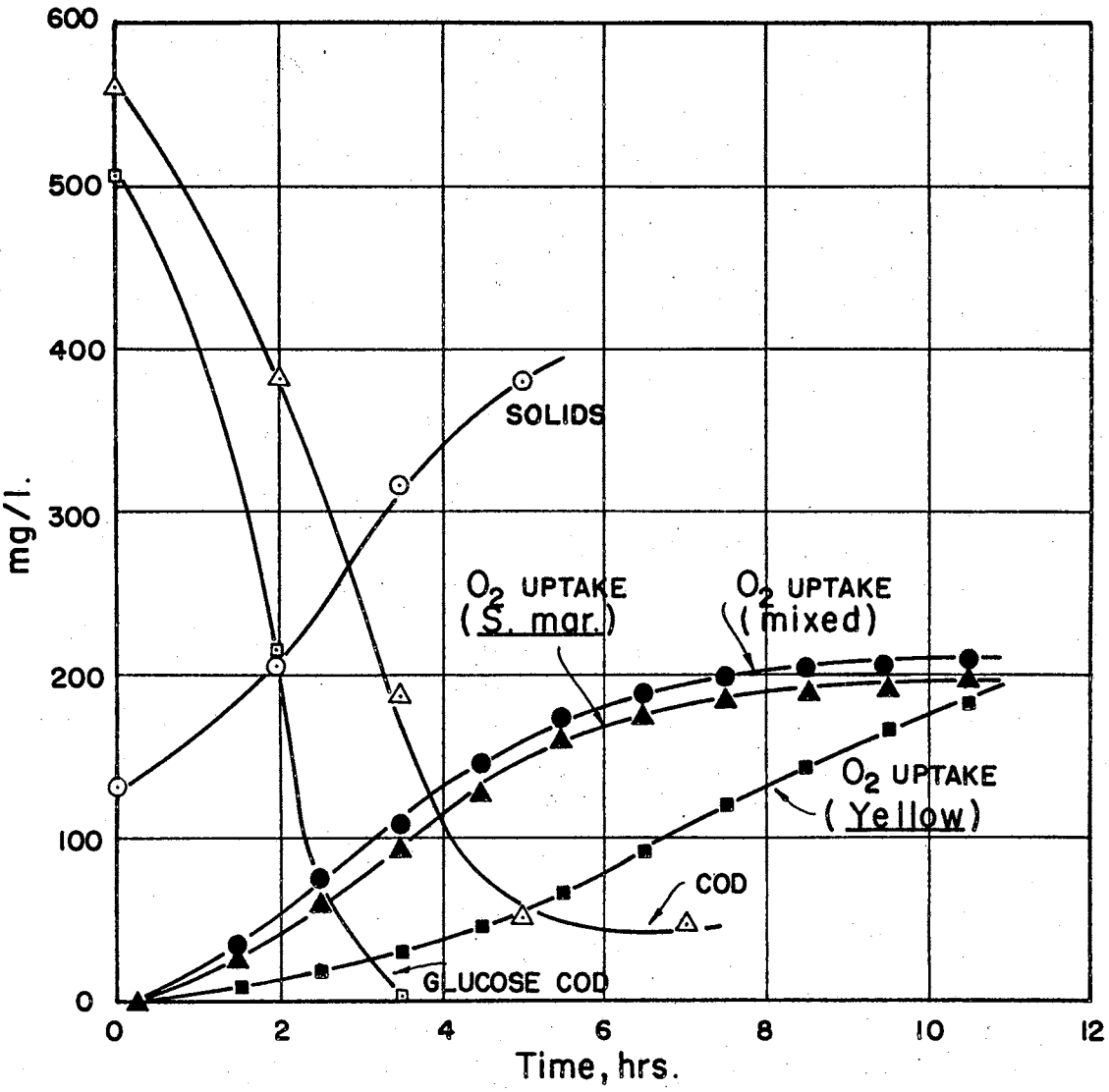

Figure 72. Biochemical response of a mixed system of Serratia marcescens and Yellow organism.

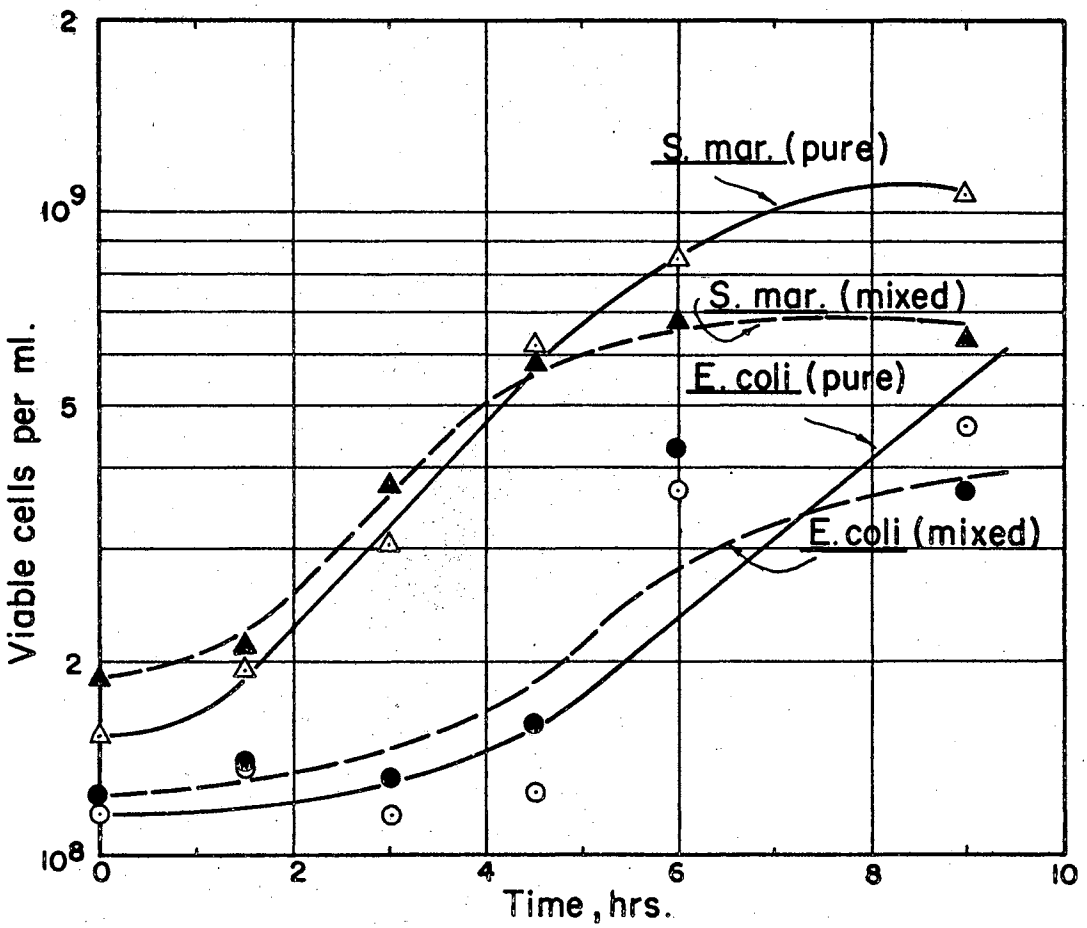

Figure 73. Viable count for Serratia marcescens and Escherichia coli in a mixed system.

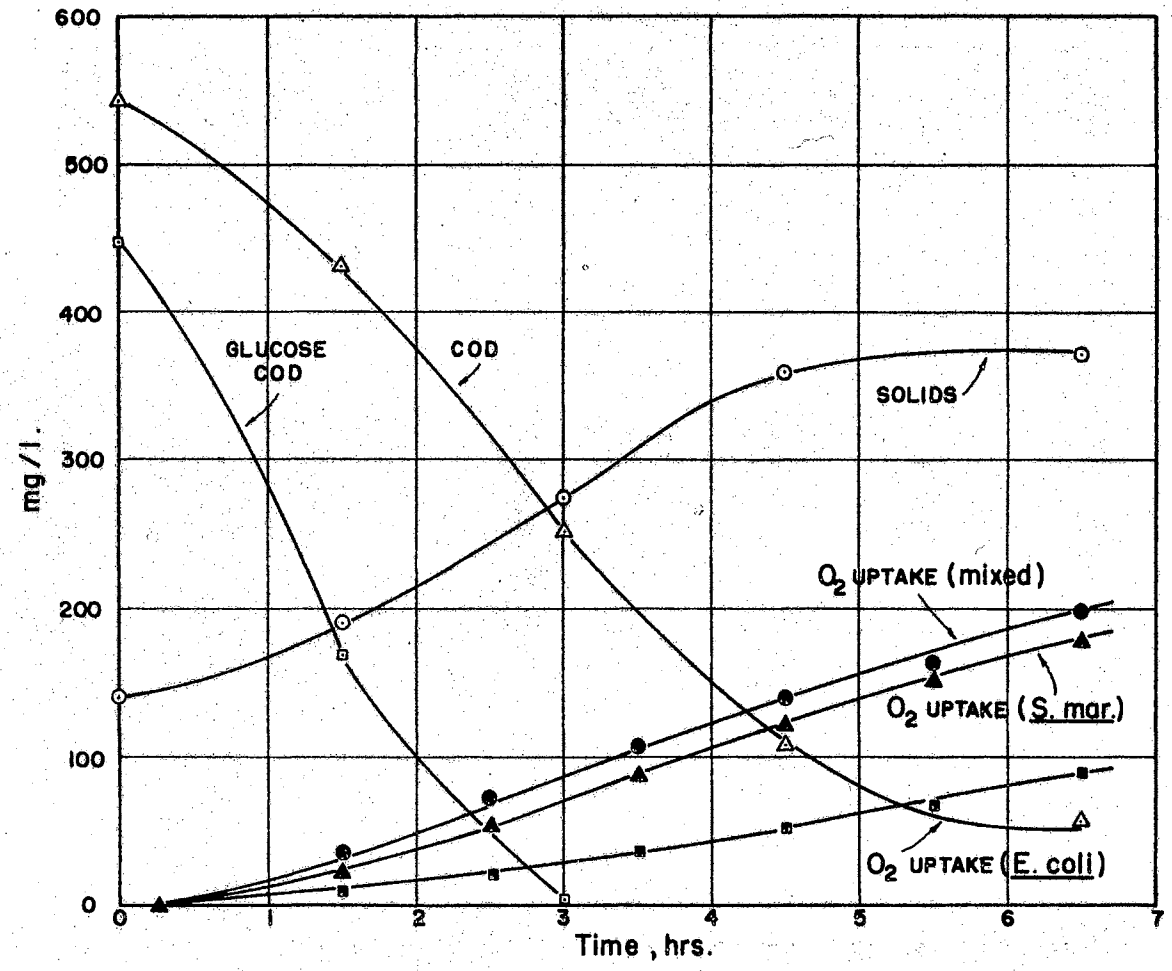

Figure 74. Biochemical response of a mixed system of Serratia marcescens and Escherichia coli.

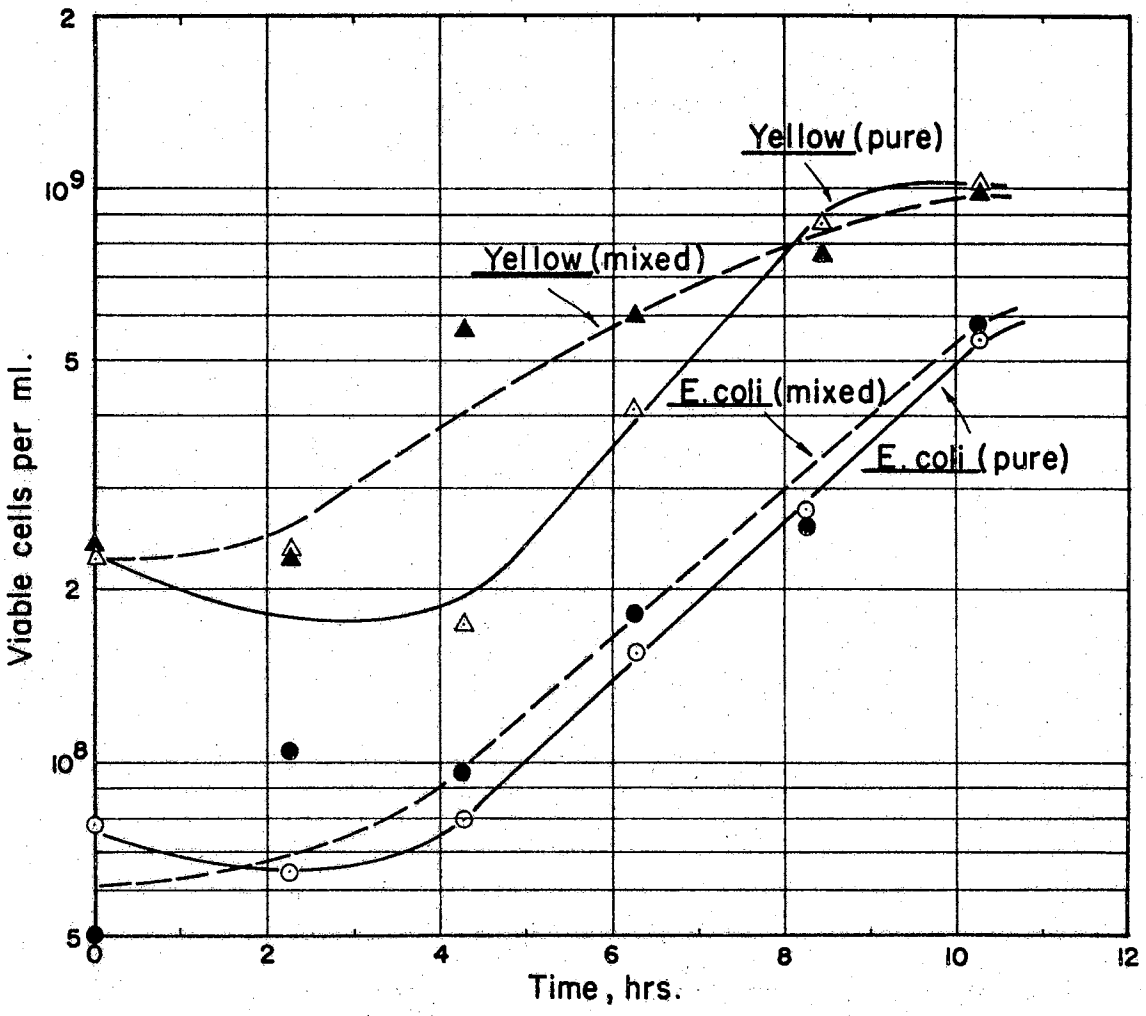

Figure 75. Viable count for Yellow organism and Escherichia coli.

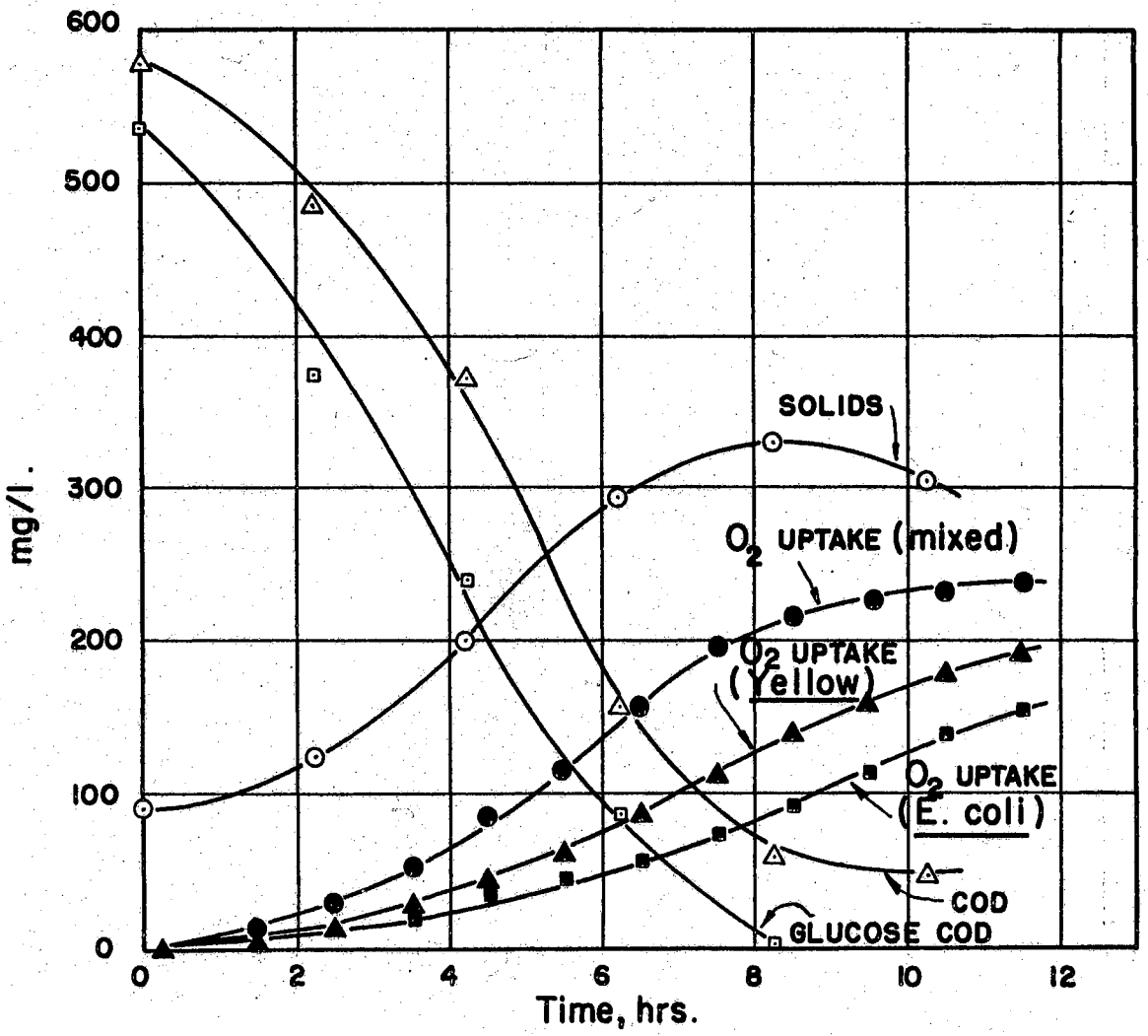

Figure 76. Biochemical response of a mixed system of Yellow organism and Escherichia coli.

indicated by the oxygen uptake of the mixture which is somewhat larger than the sum of the uptakes of both organisms up to $6\frac{1}{2}$ hours. From Figure 75 it may be seen that the viable numbers of each organism in the mixture were very close to those for each organism alone at the end of the experimental run, although Yellow organism in the mixed system appeared to grow better during the first hours of the experiment. Table VI includes the cell yields obtained in these experiments.

Analysis

From the data shown it may be surmised that those organisms in the first group of experiments participated in direct competition for the available substrate without any apparent antagonistic effects. As might have been predicted, the mixtures were very active and removed substrate at a very rapid rate. The μ_{max} values of the cultures used were very close together; no gross effect on predominance patterns was expected and none was observed.

The three experiments in the fast medium-fast group that included Serratia marcescens showed a remarkable uniformity of results (Figures 59-64). In these three experiments it is evident that no antagonism was present and that the primary mechanism in their growth response was direct competition for the available substrate. However, in these cases there was some difference between the μ_{max} values for each pair of organisms, with the result that the slower-growing organism, Serratia marcescens, was depressed in its growth to a noticeable degree. An interesting

TABLE VI

CELL YIELD IN MIXED BATCH EXPERIMENTS USING TWO ORGANISMS AND GLUCOSE AS SUBSTRATE

	mg solids/mg glucose		
	Yield A	Yield B	Yield mixed
Figures 55-56 A *Pseudomonas aeruginosa* B *Escherichia intermedia*	0.480	0.680	0.500
Figures 57-58 A Blue organism B *Pseudomonas aeruginosa*	0.697	0.482	0.630
Figures 59-60 A *Serratia marcescens* B Blue organism	0.390	0.610	0.480
Figures 61-62 A *Serratia marcescens* B *Escherichia intermedia*	0.447*	0.578*	0.472
Figures 63-64 A *Serratia marcescens* B *Pseudomonas aeruginosa*	0.470	0.470	0.490
Figures 65-66 A *Escherichia intermedia* B Yellow organism	0.578*	0.473*	0.680
Figures 67-68 A Blue organism B Yellow organism	0.697*	0.473*	0.676
Figures 69-70 A *Pseudomonas aeruginosa* B *Escherichia coli* K-12	0.450	0.424*	0.489
Figures 71-72 A *Serratia marcescens* B Yellow organism	0.478	0.462	0.496
Figures 73-74 A *Serratia marcescens* B *Escherichia coli* K-12	0.447*	0.424*	0.460
Figures 75-76 A *Escherichia coli* K-12 B Yellow organism	0.420	0.460	0.472

*Yield obtained from previous experiments

growth response is noted from Figure 67, where Yellow organism in mixed culture grew very little, the effect in this case being so severe that it could have been the result of antagonism.

In the case of Escherichia intermedia and Yellow organism, it was seen from Figure 65 that the initial numbers of Escherichia intermedia were very low in comparison with those of Yellow organism, but that the numerical ratio of organisms was greatly reduced at the end of the growth period. Also noted was a decrease in the numbers of Yellow organism after all substrate had been removed from the medium; however, it is believed that this effect was due, not to further antagonism, but to flocculation of the cells.

From the experiment performed with a fast grower, Pseudomonas aeruginosa, and a slow grower, Escherichia coli (Figure 69), it can be seen that a prediction of predominance based on direct substrate competition with μ_{max} as a parameter would be essentially accurate. No antagonistic effect was detected in this experiment. In the case of the Serratia marcescens-Yellow organism mixture (Figure 71) it was noted that no growth was observed with Yellow organism. Clearly, prediction of relative numbers on the basis of μ_{max} values would not suffice for this system. These results may be explained by an antagonism of Serratia marcescens for Yellow organism; however, it is to be remembered that the latter organism is a very difficult one

to work with and that the viable count response shown may not represent the true situation in the mixture because of the tendency of the organism to flocculate and to become attached to the walls of the reaction vessel.

An examination of the viable count results of the two experiments involving mixtures of slow and medium-fast growing organisms (Figures 73 and 75) does not reveal any particular growth advantage for the organisms tested. However, it is of interest to note that in mixed systems, a reduction of the lag phase in the growth of the individual organisms may sometimes be observed, as can be seen in Figure 75 for the Yellow organism.

Over-all analysis of the predominance patterns and their relation to predictions based on μ_{max} values for each specific organism allows the tentative conclusion that μ_{max} values may be of great importance in the prediction of the predominating species in mixed systems. In systems of two organisms with similar μ_{max} values, or where the difference in magnitude is not significant, generally no unexpected predominance ratios were noted. If there was an appreciable difference in the respective maximum growth rates of the organisms, the one with the highest μ_{max} would generally predominate. A study of the results of these experiments did not allow for conclusions to be drawn concerning the influence of K_s in predominance changes, other than the observation that it did not appear to be of significance at the substrate levels utilized. Any

prediction concerning predominance patterns based on relative μ_{max} values must be limited to cases where no antagonistic relationships are prevalent.

In reference to the activity of the bacterial systems, as measured by oxygen uptake, it has been noted that in almost all cases investigated the oxygen uptake of the mixed culture was slightly larger than the sum of the oxygen uptakes of the individual pure cultures. This small increase in oxygen uptake may be attributed to the fact that in most cases the seeds of the mixed cultures contained more viable cells than than the pure culture controls. Greater cell numbers would result in a greater uptake of oxygen as registered in the pure and mixed culture oxygen curves.

In the batch experiments, a possible antagonistic relationship was noted with the Yellow organism and Serratia marcescens system (Figure 71) and with the Yellow organism and Blue organism system (Figure 67). In both experiments growth of Yellow organism was very limited in the mixed culture. No other cases of antagonism or deleterious relationships were noted in the experiments in this section.

It is interesting to note that in the mixed system experiments in which Escherichia intermedia was present, no oxygen uptake plateau was detected (Figures 62 and 66). It seems likely that under the experimental conditions, i.e., the competitive situation, the organism did not have time to acclimate to its own intermediates before they were

assimilated by the second organism in the mixture. Also interesting is the observation that in mixed systems where Yellow organism was present, few metabolic intermediates were produced (Figures 66, 68, and 76). It has been noted previously that Yellow organism (Figure 47) did not elaborate large amounts of intermediates, but it now seems apparent that it can assimilate some of the intermediates excreted by the second culture in the mixture.

2. Mixed Cultures. Three Organisms on Glucose

Experiments were carried out by mixing cultures of three organisms in a single vessel. Viable counts were measured, during growth, for the mixed system as well as for pure culture controls. Glucose at 1000 mg/l concentration was used as carbon source.

Figure 77 shows the results of an experiment with Blue organism, Serratia marcescens, and Pseudomonas aeruginosa. Growth of Serratia marcescens was depressed in the mixed system. Blue organism, which grew the most rapidly in pure culture, remained close to its pure culture viable numbers. Pseudomonas aeruginosa remained low in numbers although its lag period was somewhat decreased in the mixture.

The results of an experiment with Yellow organism, Escherichia intermedia and Serratia marcescens are shown in Figure 78. The viable numbers of Serratia marcescens and Yellow organism were depressed, while Escherichia intermedia remained close to its pure culture numbers after completion of substrate removal. In the mixture, Escherechia intermedia

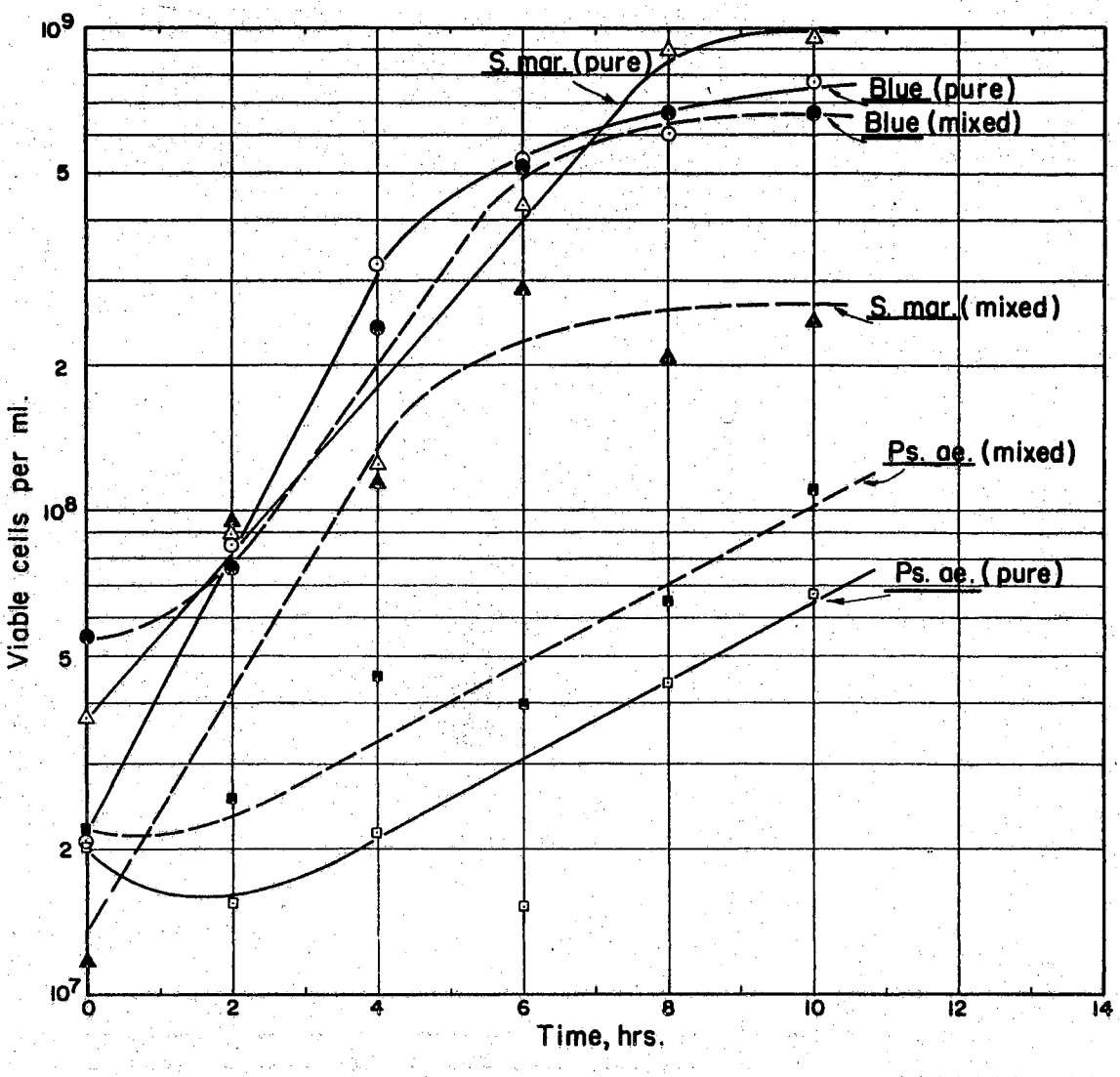

Figure 77. Viable count for Blue organism, Pseudomonas aeruginosa, and Serratia marcescens in a mixed system.

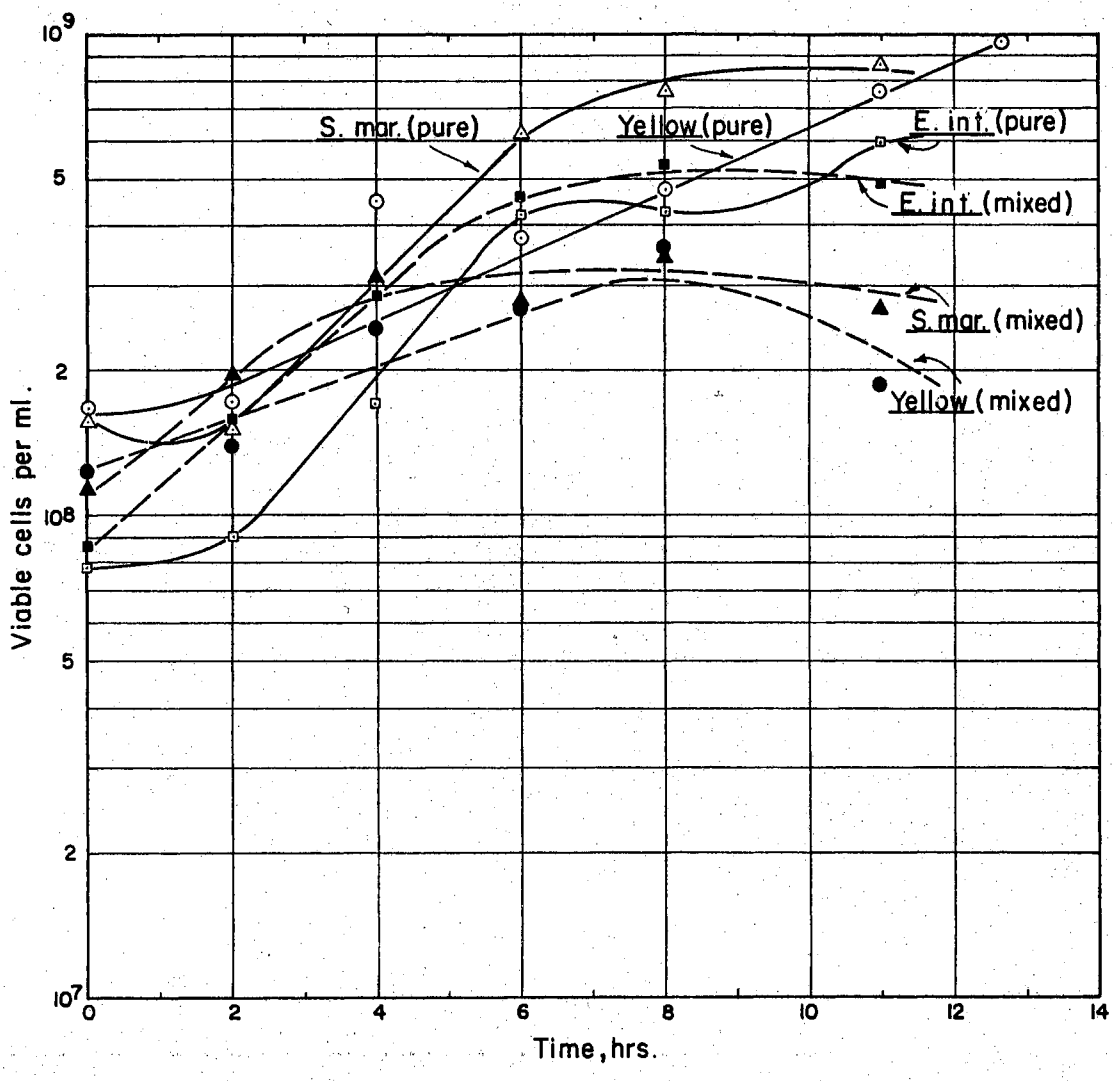

Figure 78. Viable count for Serratia marcescens, Escherichia intermedia, and Yellow organism in a mixed system.

did not evidence the lag it exhibited in the pure culture control.

Figure 79 shows the results of an experiment with Escherichia coli, Yellow organism, and Serratia marcescens. It can be observed that the viable counts of all three were depressed.

Analysis

From these experiments it can be seen that predominance predictions in batch systems based on μ_{max} values can be extended to include systems of three organisms. The experiments illustrated in Figures 78 and 79 show curves and final population densities related to the μ_{max} values for the organisms. In Figure 77 it was noted that Pseudomonas aeruginosa was subject to a lag period for which no explanation has been found. This resulted in a lower number of this species than would have been predicted. This experiment serves to illustrate the difficulties of predominance prediction, since it is obvious that lag periods such as the one experienced with Pseudomonas aeruginosa could invalidate the predictions.

It is important to note that the relative population densities of the original seed in a batch culture could be a determinative factor in the total population distribution at the end of the growth period. In cases where the initial seed counts differ, the actual population relationship at the end of the growth period may change, although the predominance profile in a qualitative sense may be expected to

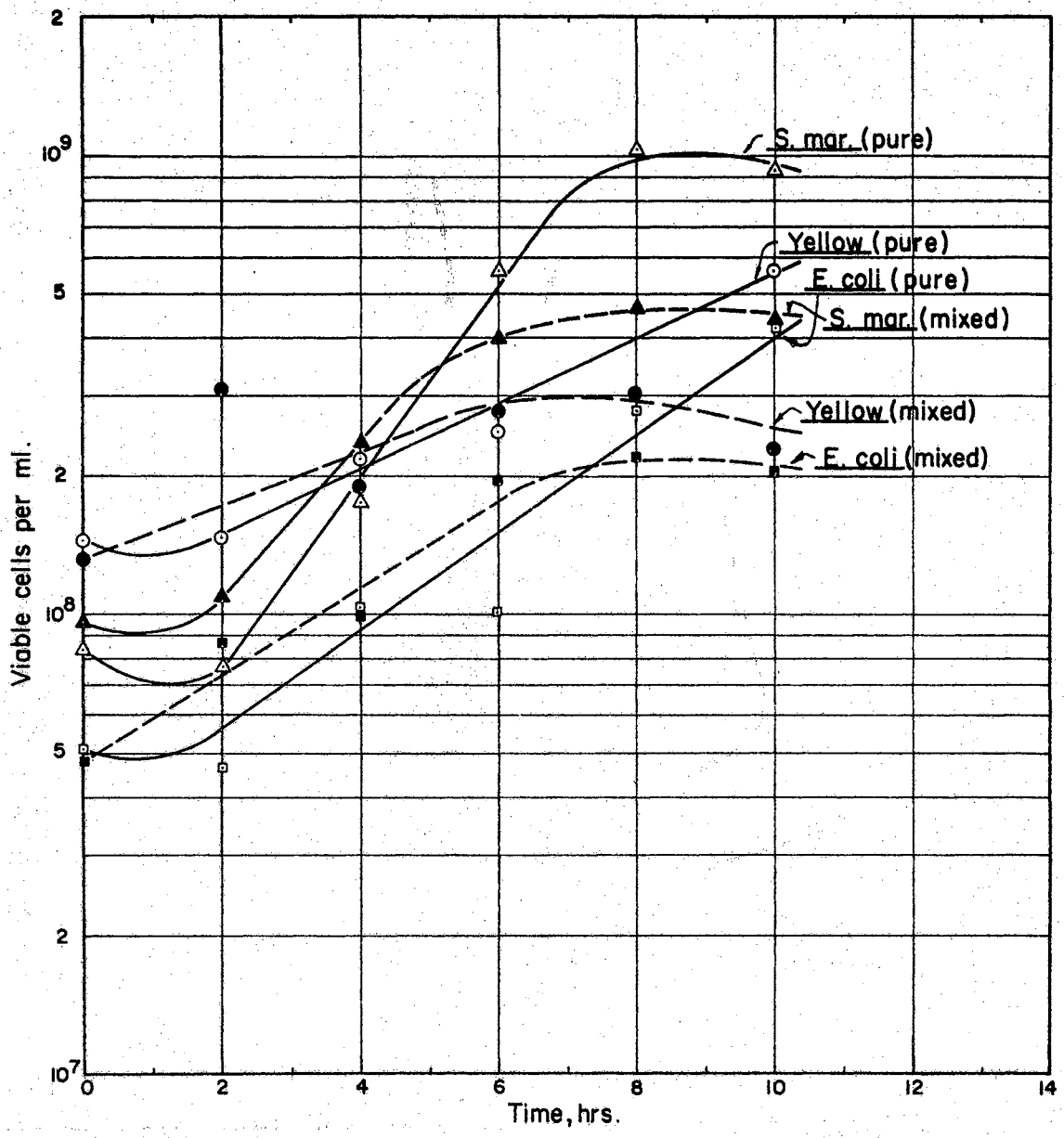

Figure 79. Viable count for Serratia marcescens, Yellow organism, and Escherichia coli in a mixed system.

remain the same as predicted by consideration of the μ_{max} values. In regard to this matter, it appeared important to investigate the behavior of a system in which organisms with different μ_{max} values and with a wide disparity in initial numbers were grown in association. Such a system, with Serratia marcescens and Escherichia coli was studied, and the results are shown in Figure 80. The experiment serves to demonstrate how rapidly changes in predominance can be brought about if the conditions for such changes are favorable.

3. Mixed Culture Combination of Two Organisms Growing on Various Substrates

In these experiments, two organisms were placed together and fed a single carbons source that could not sustain growth of one of the cultures. Substrate at 700 mg/l carbohydrate was used in the mixed systems.

Figure 81 shows the results of an experiment with Serratia marcescens and Pseudomonas aeruginosa, using sorbitol as substrate. Pseudomonas aeruginosa could not be acclimated to sorbitol and did not grow in its presence, as shown by the control curve. In the mixed culture, however, a slight increase in numbers of Pseudomonas aeruginosa was observed. After the experiment had been concluded, the viable count was about twice that of the initial seed.

Figure 82 shows the results for a system of Serratia marcescens and Pseudomonas aeruginosa growing on sucrose. It is seen that Pseudomonas aeruginosa grew on sucrose in

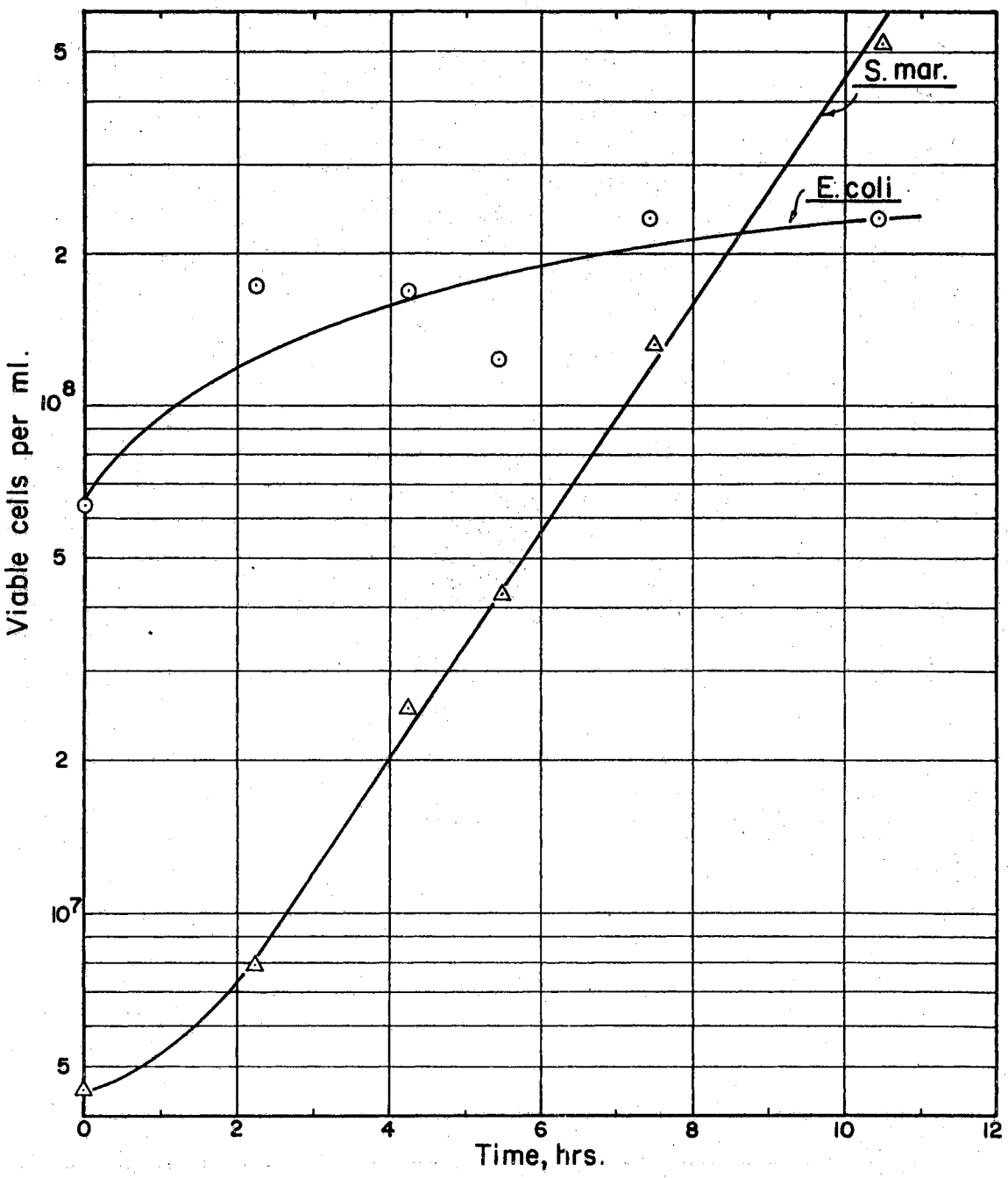

Figure 80. Viable count for Serratia marcescens and Escherichia coli in a mixed system.

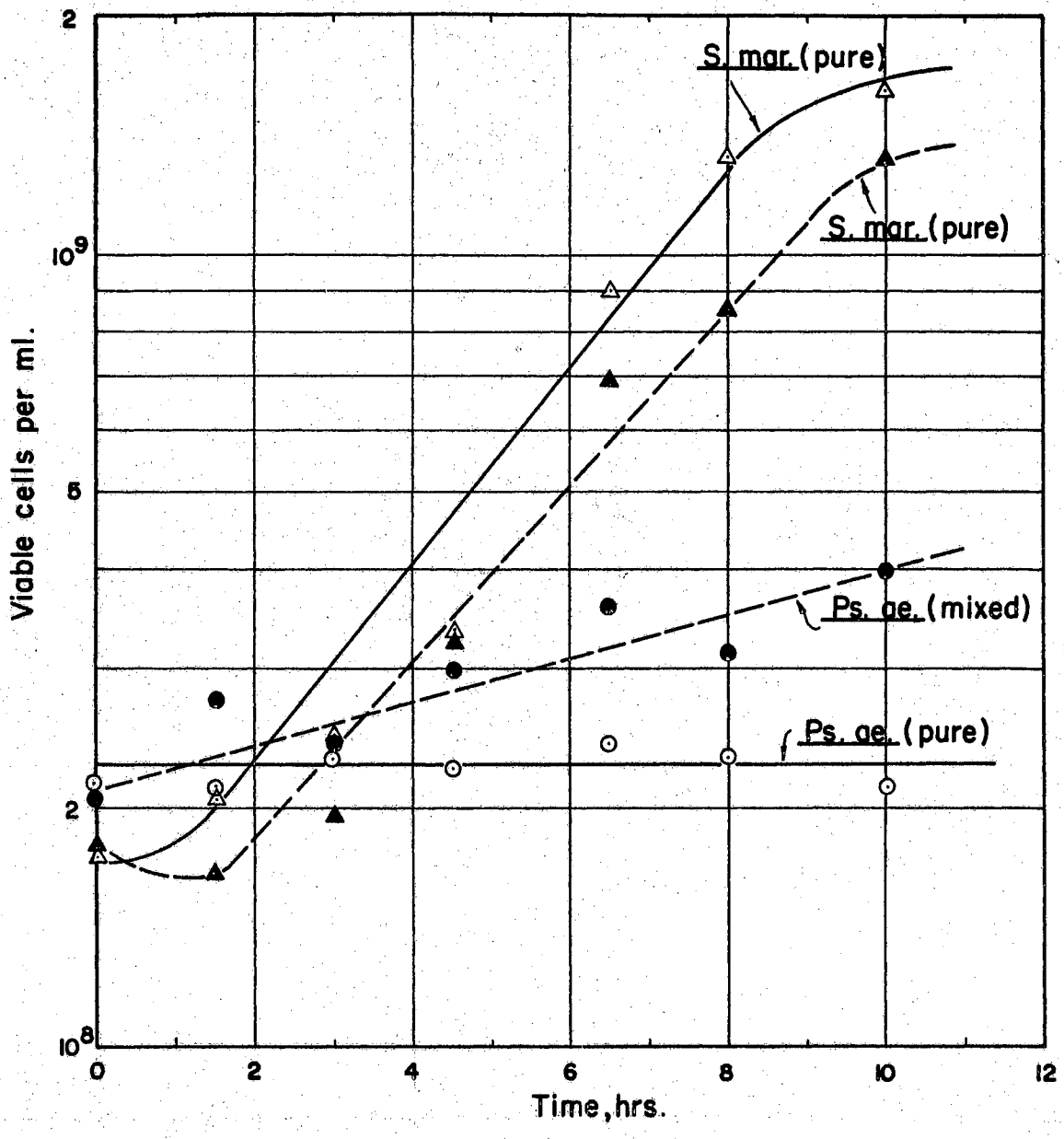

Figure 81. Viable count for Serratia marcescens and Pseudomonas aeruginosa in a mixed system with sorbitol as substrate.

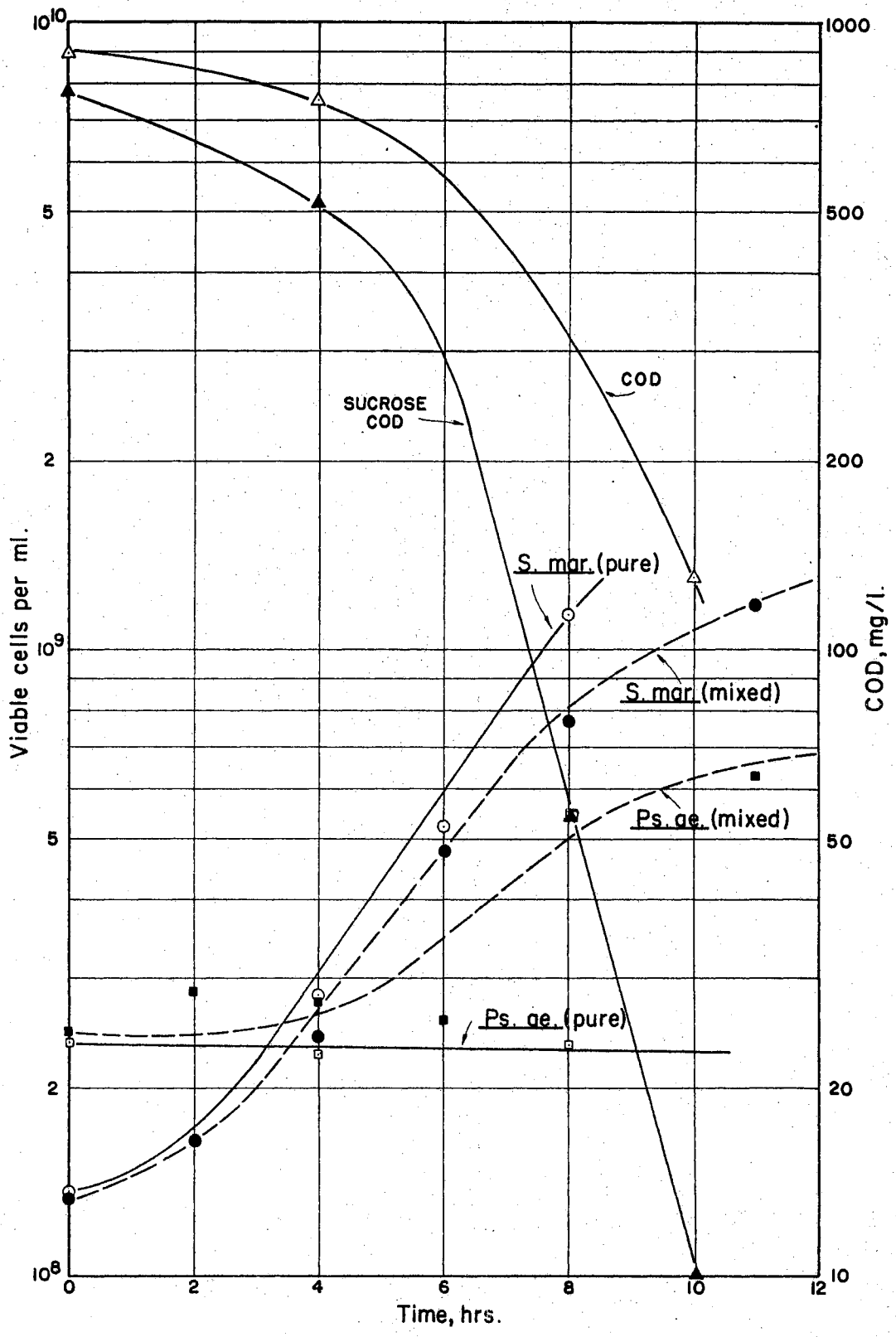

Figure 82. Viable count and biochemical response of Serratia marcescens and Pseudomonas aeruginosa in a mixed system with sucrose as substrate.

the mixed population with Serratia marcescens. COD and sucrose COD curves show the production of considerable amounts of metabolic intermediates.

A number of other experiments along the same lines as the two reported above were attempted. These included Serratia marcescens and Yellow organism on sorbitol, Escherichia intermedia and Pseudomonas aeruginosa on sorbitol, Blue organisma and Pseudomonas aeruginosa on sorbitol, Blue organism and Yellow organism on sorbitol, Blue organism and Pseudomonas aeruginosa on sucrose, Serratia marcescens and Pseudomonas aeruginosa on ribose. None of these experiments indicated that the second organism could grow in association with the first, with the possible exception of an experiment using Blue organism and Pseudomonas aeruginosa on sucrose where Pseudomonas aeruginosa grew to a very limited extent.

Analysis

These experiments show that, when in association with another organism which can use a particular substrate for growth, a second organism which cannot use the substrate in pure culture can register a growth response. However, based on the experiments performed, such a statement should be approached with caution in view of the numerous failures experienced in attempting to show the phenomenon using different bacteria and various substrates. It appears that such an effect is a particular response occurring only under a very restrictive set of circumstances. It is noted that

the effect was realized only with a Pseudomonas aeruginosa-Serratia marcescens system, and that the non-growing species possessed a high maximum growth rate with glucose, while Serratia marcescens grew slower on glucose. These growth rate conditions appear to be necessary if the phenomenon is to be explained by prescribing to Serratia marcescens the role of producer of intermediates while assuming that Pseudomonas aeruginosa, a fast grower, assimilates very rapidly a portion of the intermediates introduced into the medium. It may be surmised that the effect may not be duplicated using a fast grower, because it might be expected to take up the intermediates without giving an opportunity for the Pseudomonas aeruginosa to utilize the intermediates. There is some evidence in Figure 82 to substantiate this explanation. However, it might also be possible that the growth of Pseudomonas aeruginosa was not due entirely to metabolism of intermediates as a carbon source. It may be that the intermediates provided an energy source which aided in inducing enzymes to metabolize sucrose.

More difficult to explain is the Pseudomonas aeruginosa growth represented in Figure 81. The explanation involving growth on intermediates may not be valid in this case, because as was shown in Figure 50, no intermediates accumulated in the system in which Serratia marcescens was growing on sorbitol.

4. Starvation Experiments

These experiments were performed to study the behavior

of pure cultures and mixed cultures in the absence of exogenous substrate. Figure 83 shows the control experiments for pure cultures of Pseudomonas aeruginosa, Serratia marcescens, and Blue organism.

Figure 84 shows the behavior of a mixture of Pseudomonas aeruginosa and Serratia marcescens. The latter organism was observed to decrease in numbers at a higher rate than its control, while Pseudomonas aeruginosa was noted to be slowly increasing in viable numbers.

Figure 85 shows a system with Serratia marcescens and Blue organism, and Figure 86 shows a system with Pseudomonas aeruginosa and Blue organism. The curves in Figure 85 do not show a significant change in behavior when compared with their pure culture controls, while essentially no decrease in viability was observed for either of the species in the mixed system shown in Figure 86.

Analysis

Inspection of the pure culture controls (Figure 83) shows that all three cultures tested underwent a slow decrease in viability during a period of thirteen days of endogenous respiration. In one of the mixed culture experiments (Figure 85) no interaction between the two species was observed. The organisms behaved in a manner similar to that observed in the pure culture controls (Figure 83).

Results of the experiment involving a mixture of Pseudomonas aeruginosa and Blue organism (Figure 85) indicated no decrease in viable numbers as a result of

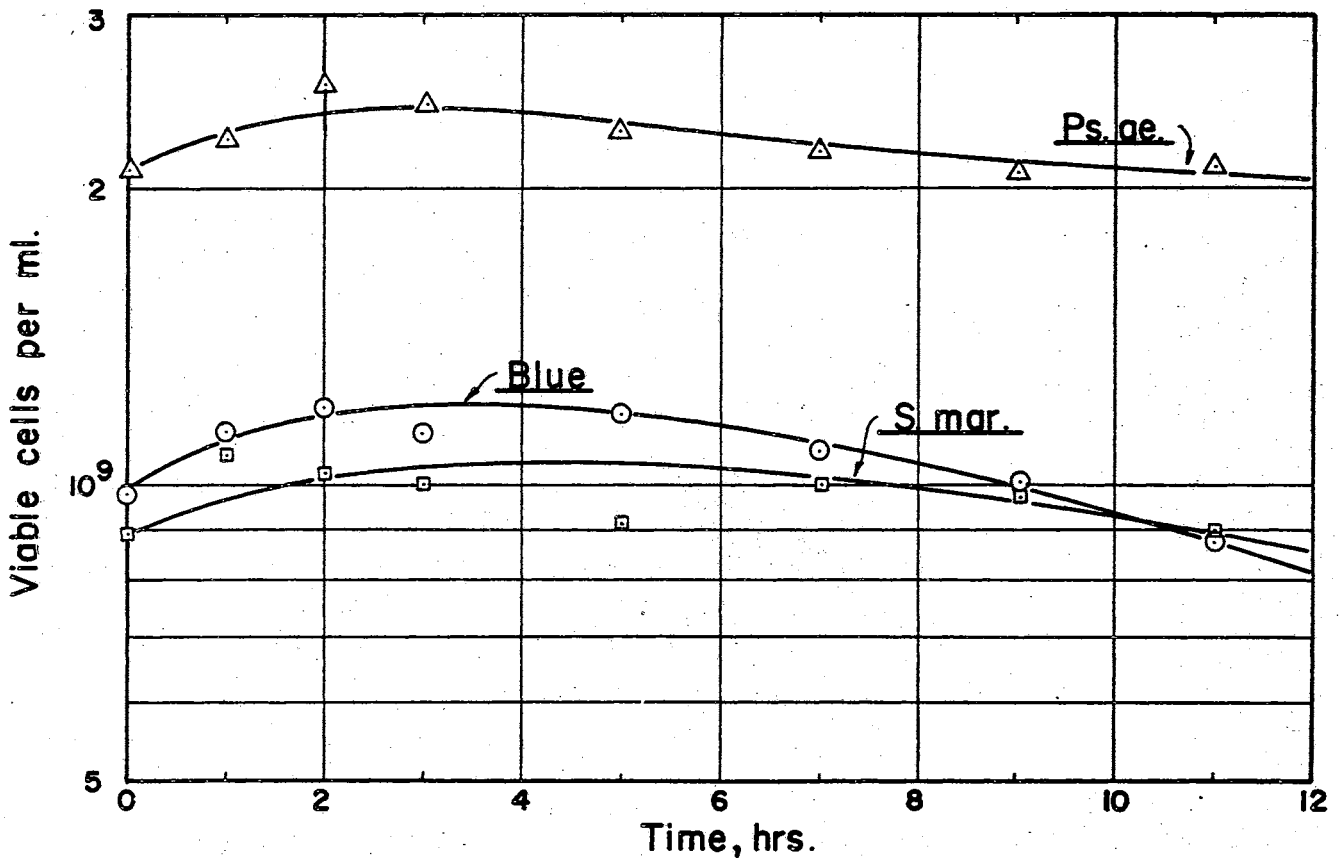

Figure 83. Starvation Experiments: Pure culture controls of Pseudomonas aeruginosa, Blue organism, and Serratia marcescens.

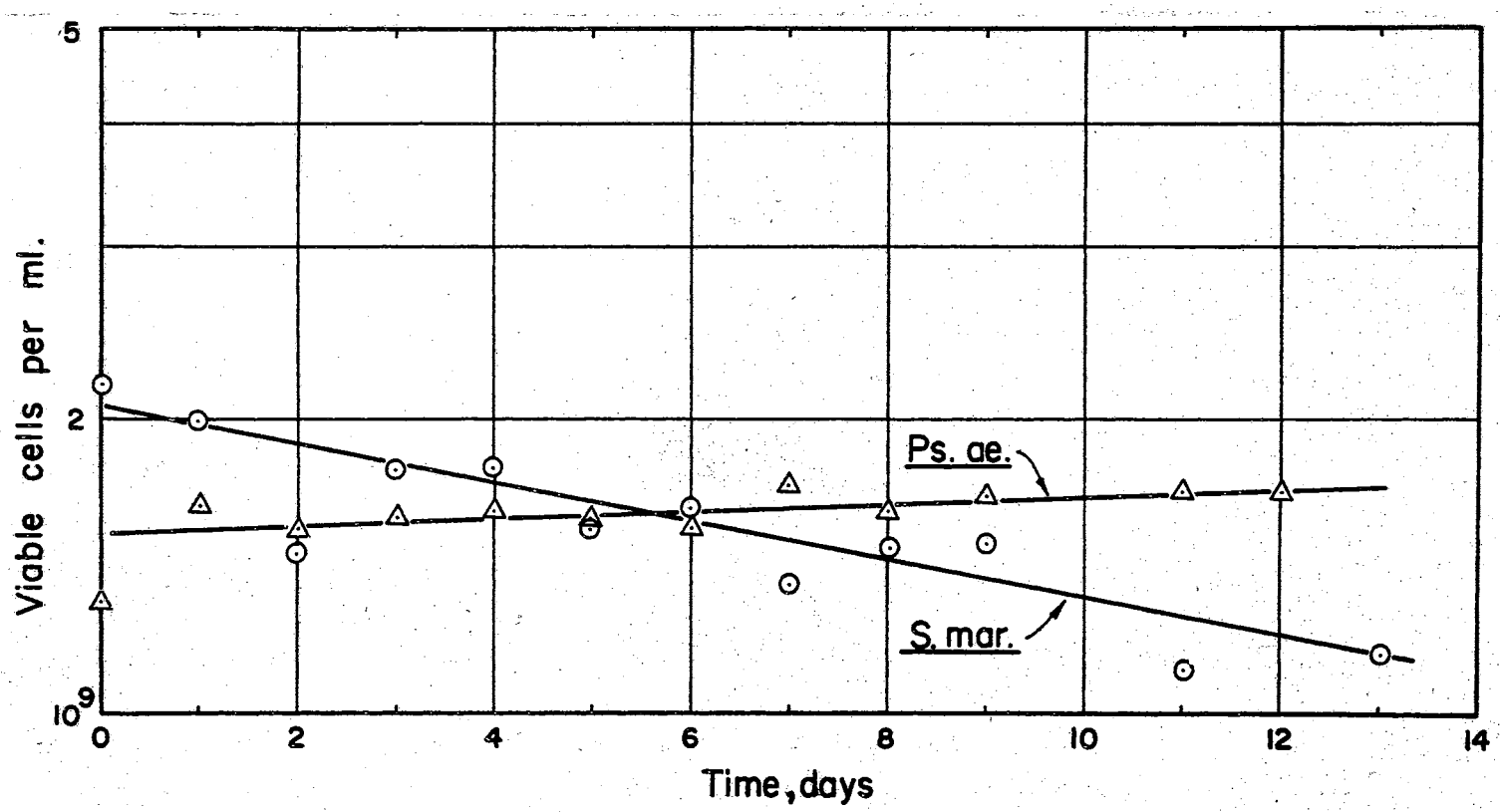

Figure 84. Starvation Experiments: Pseudomonas aeruginosa and Serratia marcescens in a mixed system.

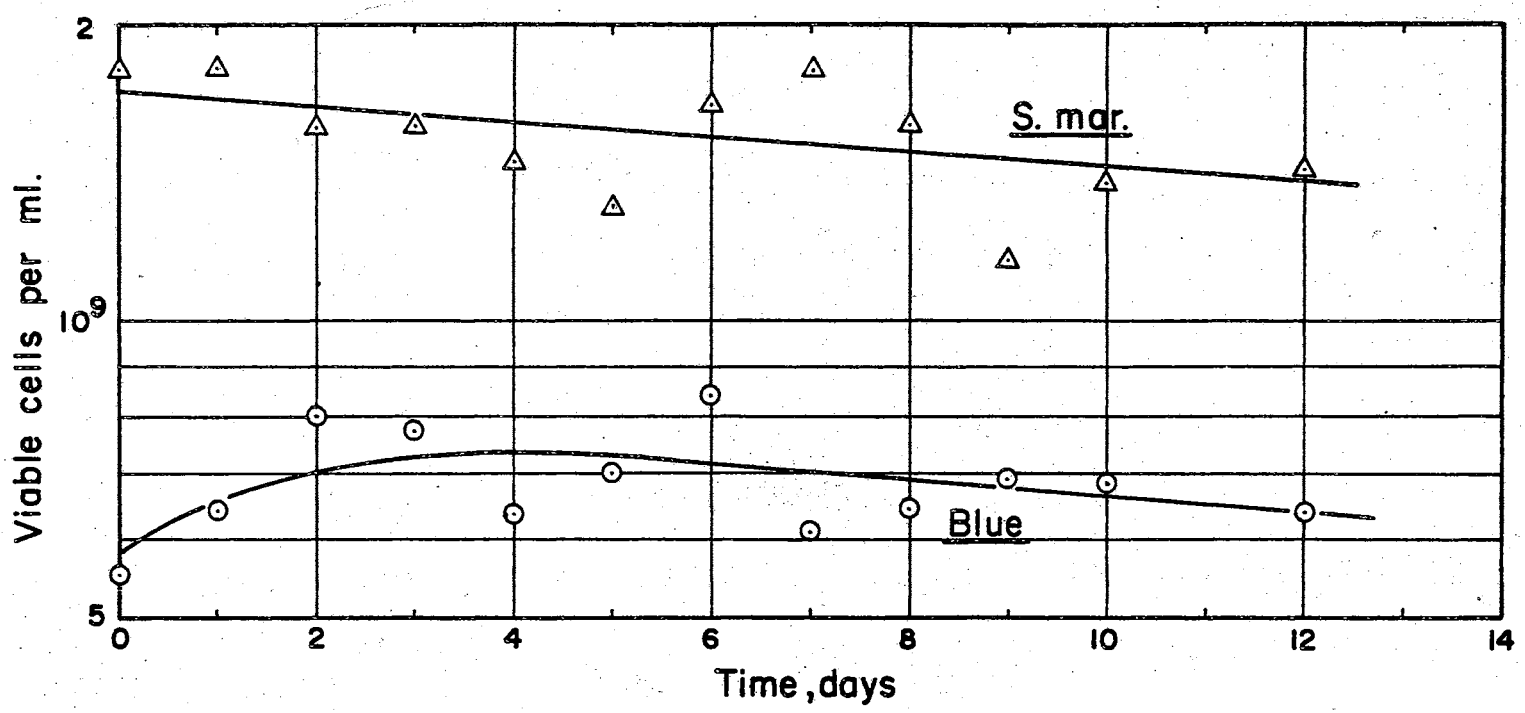

Figure 85. Starvation Experiments: Serratia marcescens and Blue organism in a mixed system.

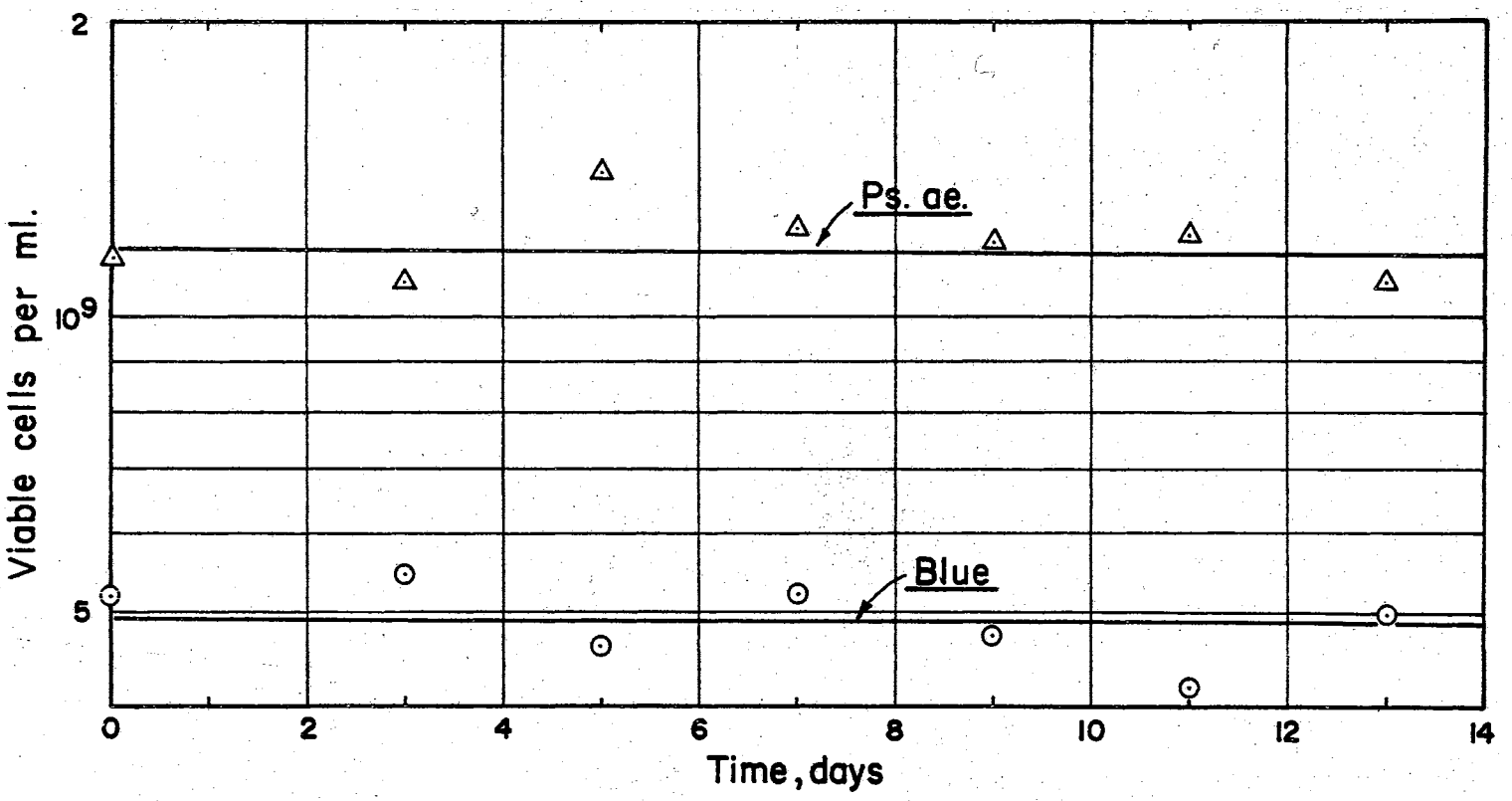

Figure 86. Starvation Experiments: Pseudomonas aeruginosa and Blue organism in a mixed system.

incubation in the absence of a carbon source. From these observations it may be surmised that the difference in the behavior of each culture in the mixed system and in the pure culture controls results from the associative relationship of both species. In this case, a beneficial relationship was developed, with the cultures in the mixed system maintaining their original levels of viable population density.

In the experiment with Pseudomonas aeruginosa and Serratia marcescens (Figure 84) the organisms in the mixture behaved in a distinctly different manner when compared with their controls. Viable numbers of Serratia marcescens decreased at a greater rate than the decrease of the same organism observed in its control, while Pseudomonas aeruginosa experienced a period of growth. Since there was no exogenous source of nutrients that could provide for such growth, it may be surmised that Pseudomonas aeruginosa obtained its carbon source from Serratia marcescens. It is possible that Pseudomonas aeruginosa interacted with the other organism in such a manner as to lyse some of the cells, and then assimilated the released nutrients for its own benefit. Such an explanation could account for the increased die-off observed in the Serratia marcescens viable count curve (Figure 84). At this point, it is interesting to note that the same mixture of organisms produced growth of Pseudomonas aeruginosa on sorbitol (Figure 81) even though the latter organism could not utilize sorbitol.

D. Continuous Flow Studies with Pure Cultures

1. Dilution Curves Using Methyl-red Dye

To check mixing conditions in the chemostat reactor, dilute-out experiments were performed with a solution of methyl-red at pH 2.0 using three different flow rates. The concentration of the dye solution was measured by optical density at a wave length of 570 mμ. Experiments were performed with and without a stirring magnet in the reactor. Figure 87 shows the theoretical and observed dilute-out curves for a chemostat without the magnet, while Figure 88 shows the same curves for the chemostat in which the liquid was being stirred by a magnet. As can be seen from the figures, the mixing conditions in the reactor were very close to the theoretical values.

2. Dilute-out with One Organism

In these experiments, pure cultures of microorganisms were grown at different dilution rates in a chemostat. Viable counts, substrate determinations (Glucostat) and volatile acid analyses were performed. Glucose was used as carbon source in all cases. The experiments were carried out by setting the pumping system at a small flow rate, resulting in a low dilution rate (D) and increasing D after steady state conditions were attained for each successive rate.

The theoretical formulations (Equations 25 and 26) in Chapter III were utilized to plot the theoretical substrate and cell density dilute-out curves. The kinetic constants

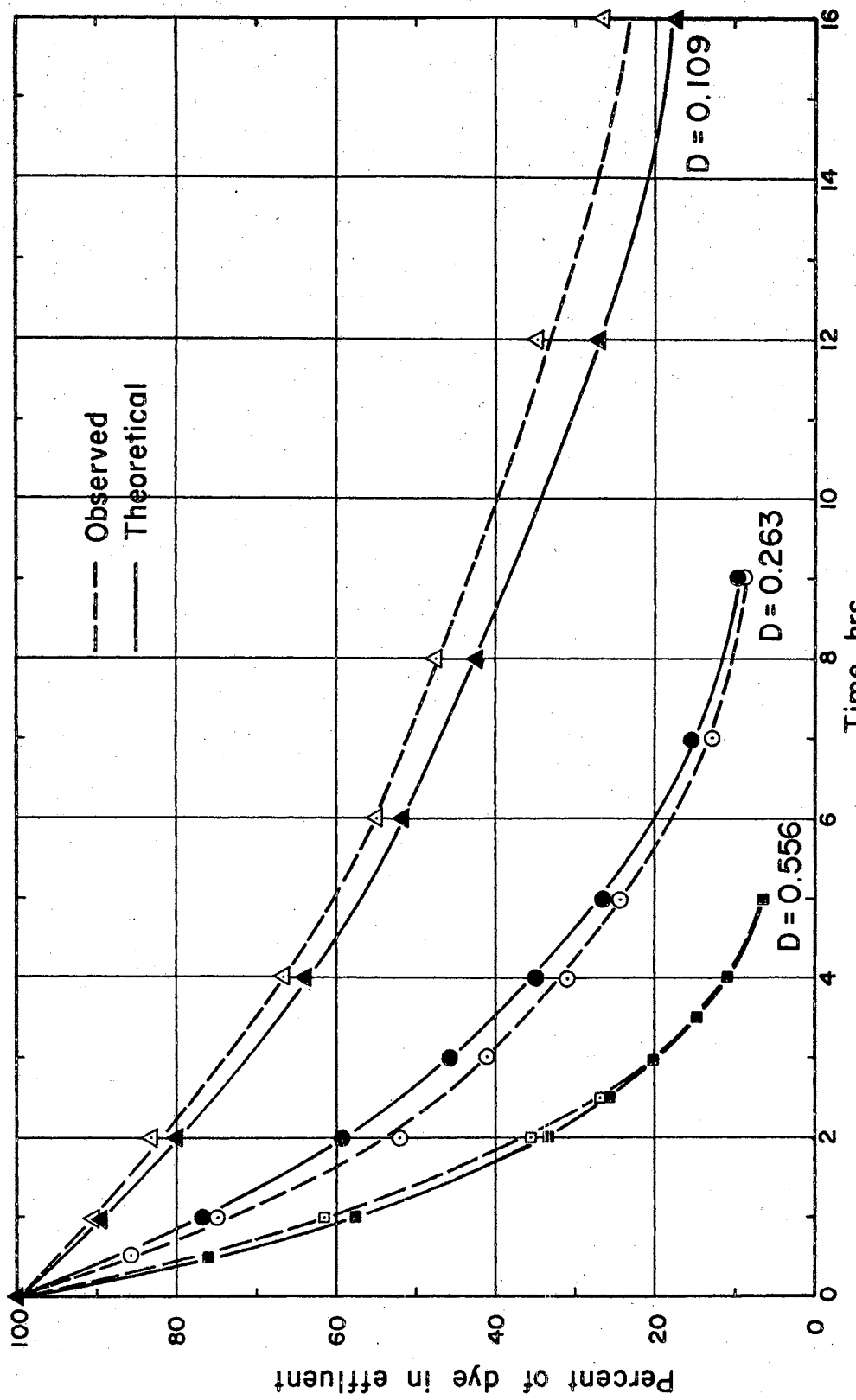

Figure 87. Degree of complete mixing in a chemostat.

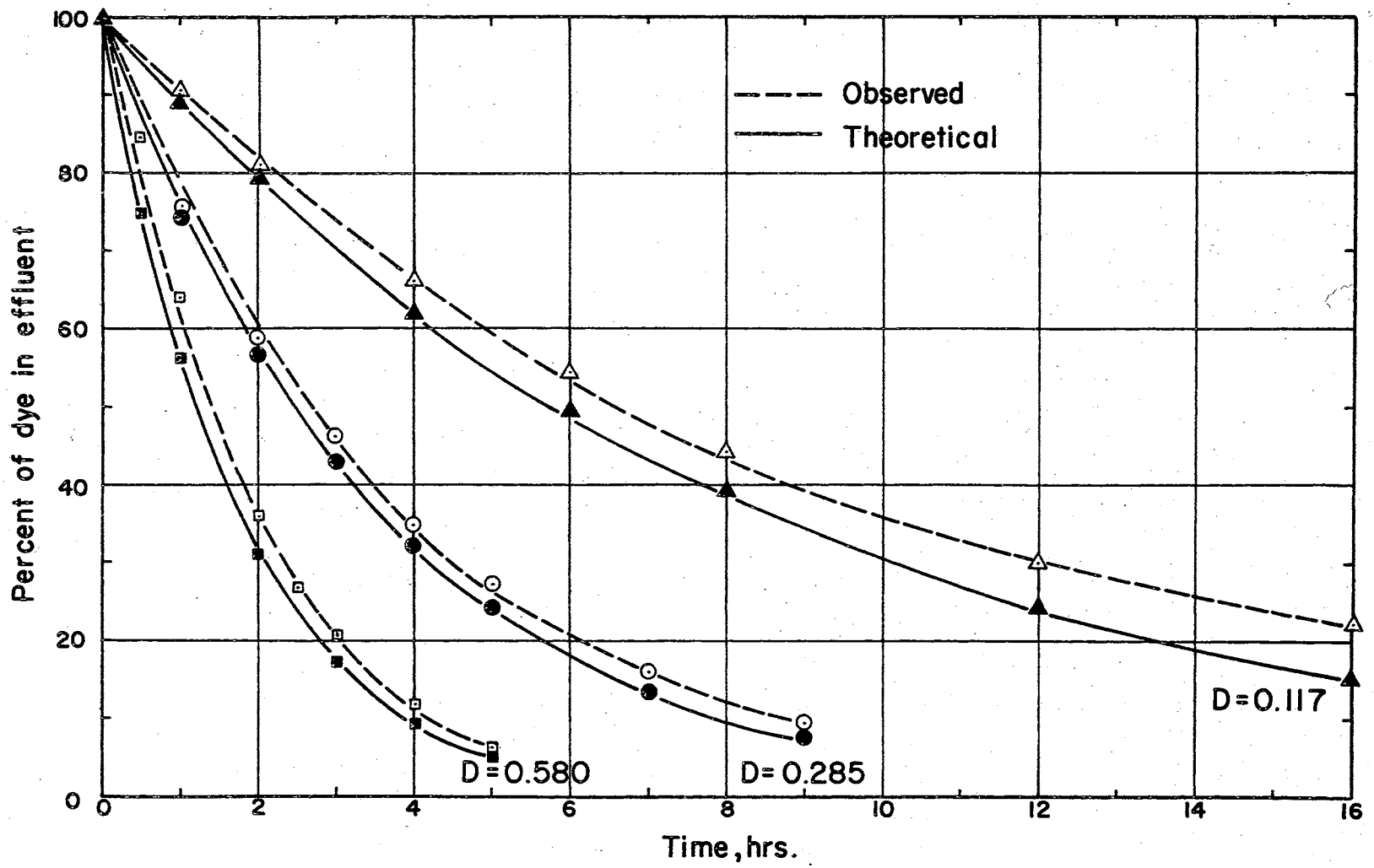

Figure 88. Degree of complete mixing in a chemostat with magnetic stirrer assistance.

used were those calculated from batch experiments as shown in Table II. In addition, theoretical curves based upon kinetic constants computed by the Lineweaver-Burk reverse plot method (Table IV) have been included in the figures (labeled "Theoretical L-B").

Figure 89 shows the results of a dilute-out experiment using Serratia marcescens. In the same figure, theoretical curves calculated for X and S have been plotted. During the experimental run it was noted that Serratia marcescens had a tendency to become attached to the walls of the reactor and to produce considerable amounts of foam. At high dilution rates the organism did not dilute out well. Volatile acid analyses were not made during this experiment.

Results of the dilute-out experiment using Pseudomonas aeruginosa are shown in Figure 90. In this experiment, where no magnetic stirrer was used to increase mixing, Pseudomonas aeruginosa showed a tendency to become attached to the walls of the reactor. The system responded to an increasing dilution rate by releasing increasing amounts of the substrate in the effluent, but the maximum effluent glucose concentration recorded was considerably less than the amount being introduced with the feed. Viable count determinations show that the observed values were lower at low D values than those computed from theoretical formulations using μ_{max} and K_s values obtained in batch studies. Also noted was the failure of the organism to dilute out to a significant extent.

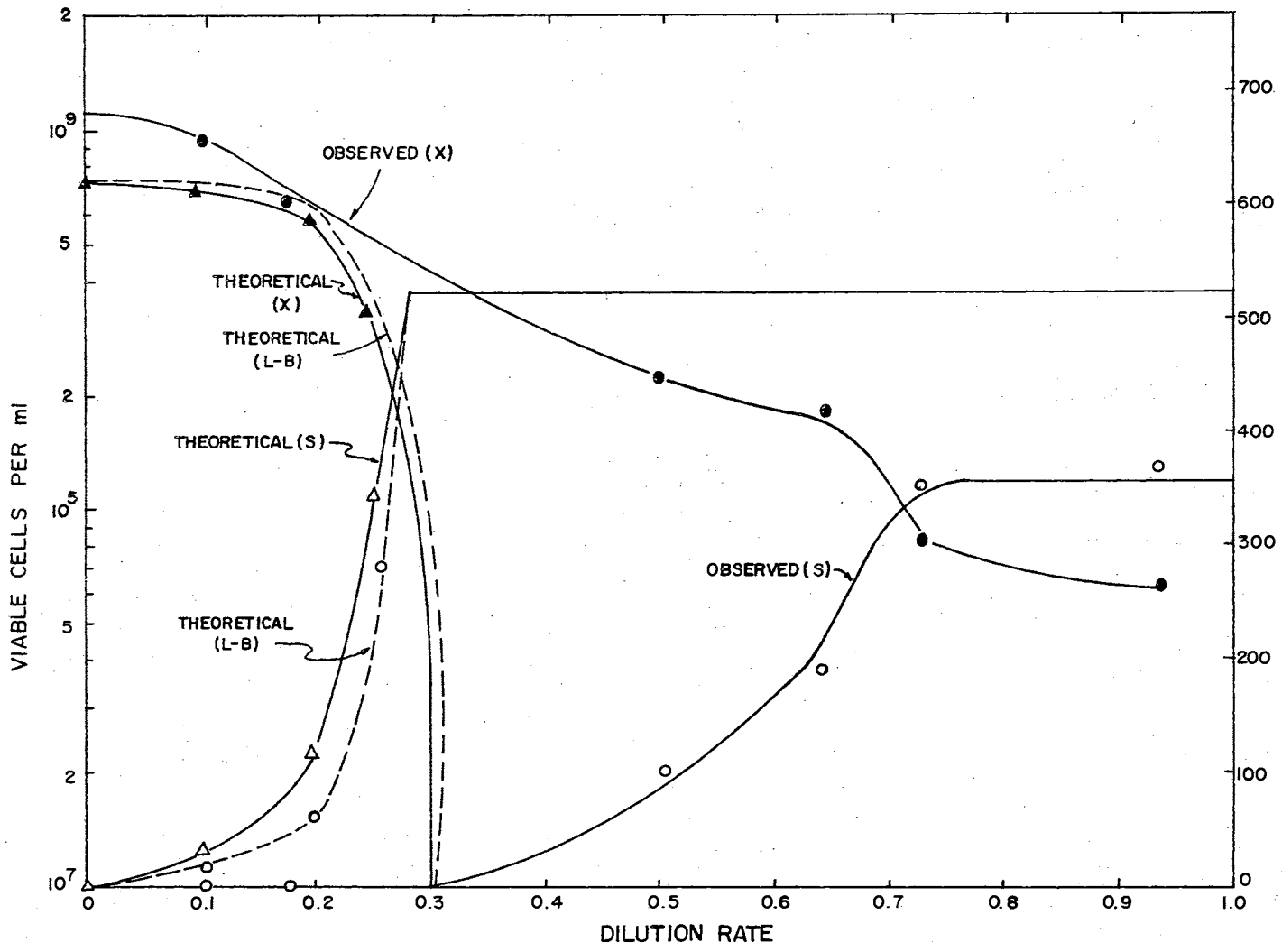

Figure 89. Theoretical and experimental dilute-out curves for Serratia marcescens.

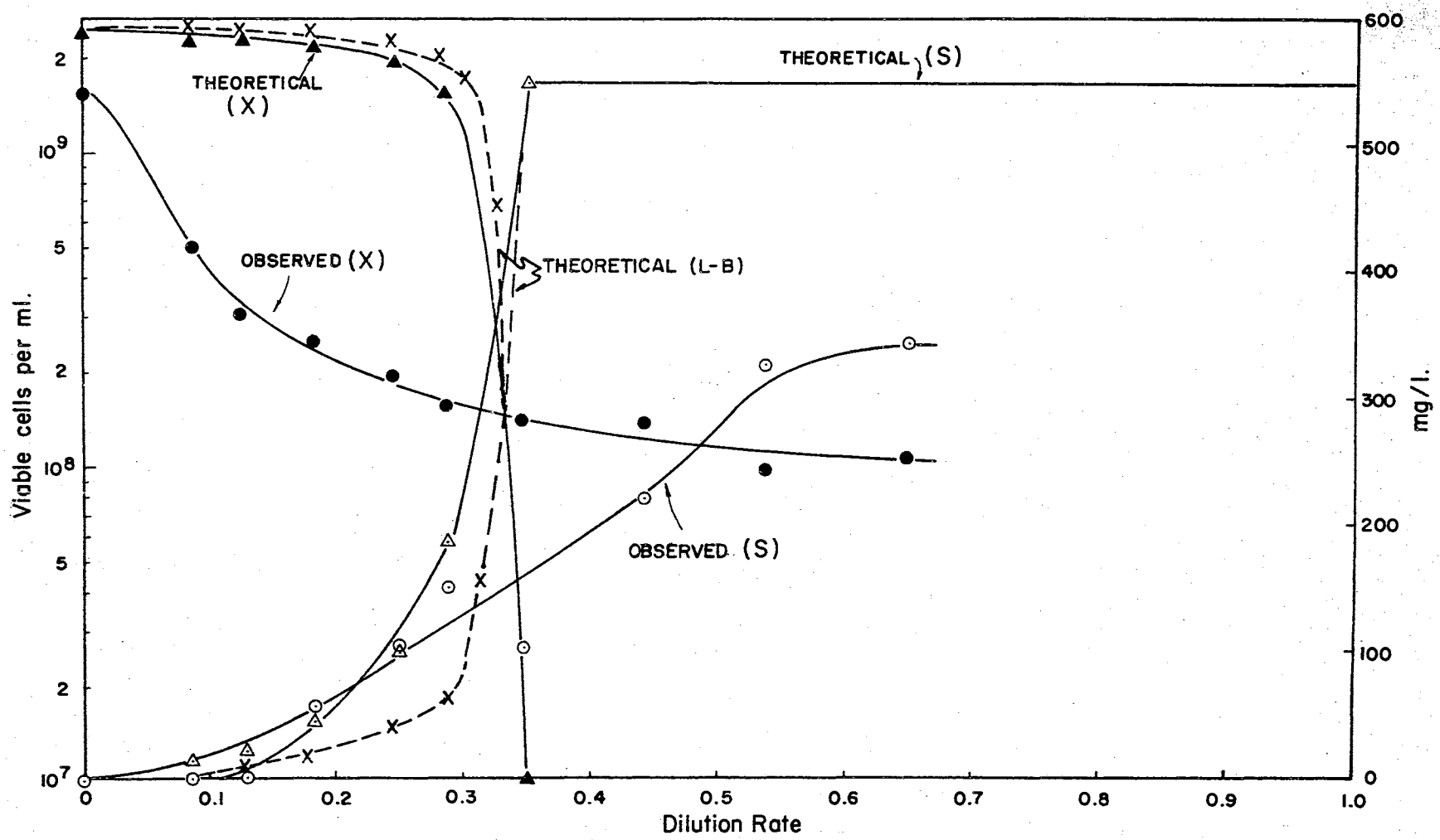

Figure 90. Theoretical and experimental dilute-out curves for Pseudomonas aeruginosa.

Figure 91 shows the results of an experiment using Blue organism. In this case, magnetic stirrer assistance was provided to increase agitation and mixing in the reactor in an effort to reduce cell attachment to the walls. Results indicate that under the experimental conditions employed, Blue organism gave a better dilute-out curve than that observed in the two previous experiments. Also noted was a better recovery of glucose in the effluent and a general location of the observed curves that more closely approximated the theoretical curves. Volatile acid analysis showed that acetic acid was being produced at all dilution rates investigated, but that its concentration tended to be higher at low D values than at high dilution rates.

Results of an experiment using Escherichia intermedia are shown in Figure 92. Magnetic stirrer assistance was utilized in this run. The data showed that the theoretical curves were reasonably close to the observed values. Acetic acid was the only volatile acid intermediate detected from gas chromatographic analysis, and its concentration was noted to be higher near the "theoretical" point of dilute-out. Again, fewer problems of foaming and cell wall attachment were noted in this experiment than in the first two experiments reported, where no magnetic stirrer was used. Magnetic stirrer agitation was considered to reduce, but not to completely eliminate, the problems presented by the physical behavior of the cells in the aerating liquor.

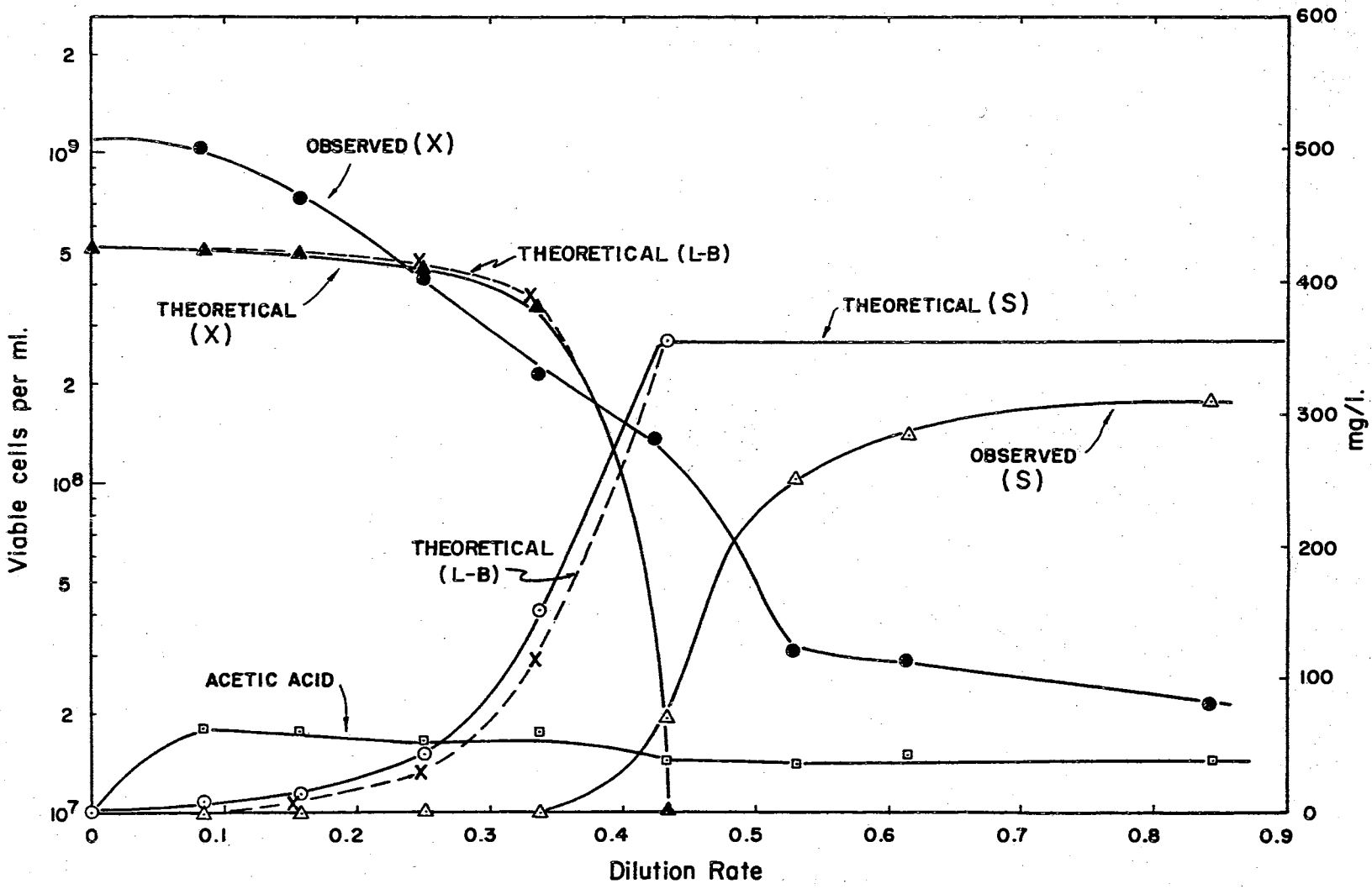

Figure 91. Theoretical and experimental dilute-out curves for Blue organism with magnetic stirrer assistance.

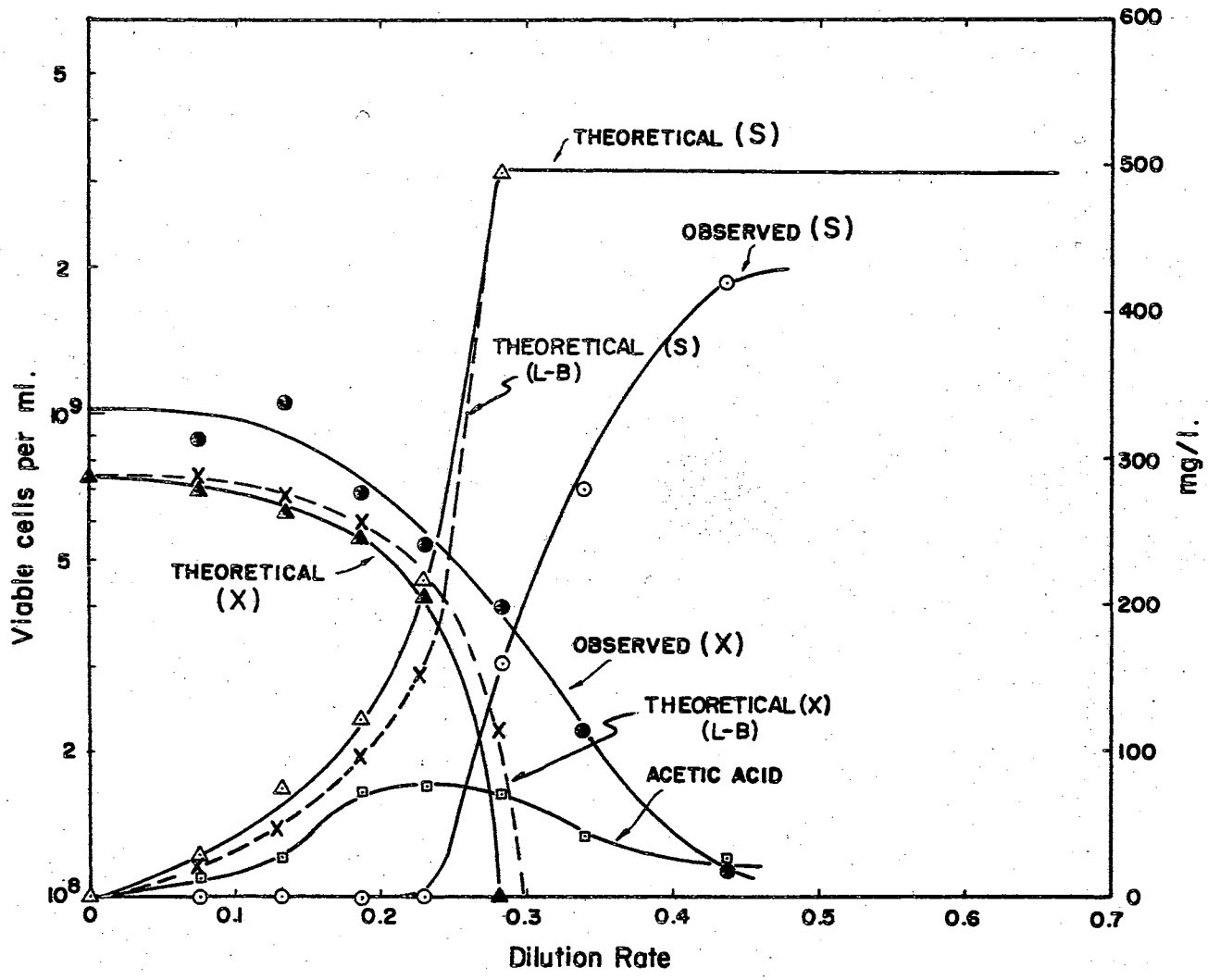

Figure 92. Theoretical and experimental dilut-out curves for Escherichia intermedia with magnetic stirrer assistance.

Analysis

If a comparison is made between the theoretical and observed dilute-out values from the experiments in which no magnetic stirrer was used (Figures 89 and 90), it can be seen that no close correlation in the values was obtained. Glucose found in the effluent was noted to be much lower, particularly in Figure 90, than that theoretically predicted at high dilution rates. Also important, and related to the glucose uptake at high D values, is the observation that cell concentration in the reactor was not reduced to a significant extent by an increase in the dilution ratio. A number of factors are considered to have been responsible for these effects, such as the tendency of the bacterial cells to attach themselves to the walls, to flocculate and precipitate to the bottom of the reactor, and to induce the production of foam. Although dilute-out experiments with dye (Figure 87) had previously indicated that a good degree of mixing was obtained in the chemostat, it was deemed necessary to increase agitation of the aerating liquor to prevent the above noted problems. This was accomplished by introducing a small magnet into the chemostat and rotating it throughout the experimental runs at a speed of 200 RPM approximately, the chemostat itself being placed upon a magnetic stirring device.

In the experiments reported in Figures 91 and 92, the glucose recovered at high D values was found to be close to that being fed into the system. In the case of Blue

organism (Figure 91), substrate recovery was as high as 87 per cent of the applied substrate, and in the case of Escherichia intermedia (Figure 92), it was 84 per cent of the applied glucose. The substrate curves in both experiments approximated the shape of the theoretical curves, but were somewhat displaced to the right.

The observed and theoretical viable count curves for both Figure 91 and Figure 92 were of similar shape. The rapid dilute-out effect took place at about the same D values as predicted, but total elimination of the cells was not accomplished. The number of cells detected in the reactor, however, were relatively few, particularly in the case of Blue organism (Figure 91). From the observed curves it is noted that at most D values the cell density values were higher than those for the theoretical curves. Such positioning of the viable cell curve can also be seen in Figure 89. From these observations it can be surmised that the continuous flow yields derived from most of these experiments were higher than those computed from batch experiments.

In certain cases, such as the experiment with Escherichia intermedia (Figure 92), if a higher μ_{max} were to be assumed than that obtained from batch experiments (i.e., 0.420), a better fit of the observed values to the theoretical relationship would be obtained. Thus the possibility must be entertained that μ_{max} in continuous flow cultures was higher than the values obtained from batch

experiments.

An over-all view of these experiments allows it to be concluded that a complete dilute-out of the cells is very difficult to obtain under the experimental conditions employed, although the performance of the system was improved by the magnetic stirring device. Long "tailing off" of viable count may be expected in experiments similar to these, resulting in the uptake of some of the glucose furnished even at high dilution rates. The comments presented above serve to emphasize the difficulties encountered in working with living cell systems of this type. Characteristics of the cultures and physical limitations of the equipment used may prevent a close correlation between theoretical and observed values.

From Figures 89-92 it may be seen that the theoretical X and S curves obtained from value computed from Monod's plot (Table II) and from reverse plots (Table IV) fall very close to each other. The conclusions derived from these experiments are not, therefore, materially changed by using the values calculated by either method.

The nature of the intermediates produced during glucose metabolism with natural heterogeneous populations has been investigated by Krishnan (116). He found that the intermediates consisted largely of volatile acids, with acetic acid being the major component. Gas chromatographic analyses of samples taken from continuous flow experiments confirm Krishnan's findings and point to acetic acid as

being the only volatile acid present in more than trace amounts.

From the Escherichia intermedia experiment (Figure 92) it can be noted that the intermediates produced were primarily acetic acid. Since the intermediates and/or end products for this organism were no different than for any other strain studied, the plateau with Escherichia intermedia must be the result of peculiar metabolic characteristics of this organism when using acetic acid. Acetic acid was excreted into the medium at all dilution rates, with an increase in quantity being observed during the actual dilute-out of the cells from the system. The same effect was observed using Blue organism (Figure 91).

E. Continuous Flow Studies with Mixtures of Pure Cultures

1. Dilute-out Experiments with Two Organisms

These experiments were carried out in a manner similar to those presented in the previous section, except that two organisms were used. Figure 93 shows the results of one such experiment using a mixture of Serratia marcescens and Blue organism. The magnetic stirrer was not used during this experiment. The viable count curves show that certain population density levels were established at low dilution rates with the viable numbers of Blue organism being higher than those of Serratia marcescens. At high dilution rates, Blue organism diluted out as expected; however, Serratia marcescens did not diminish in numbers. Visual inspection of the reaction vessel during the experimental run revealed

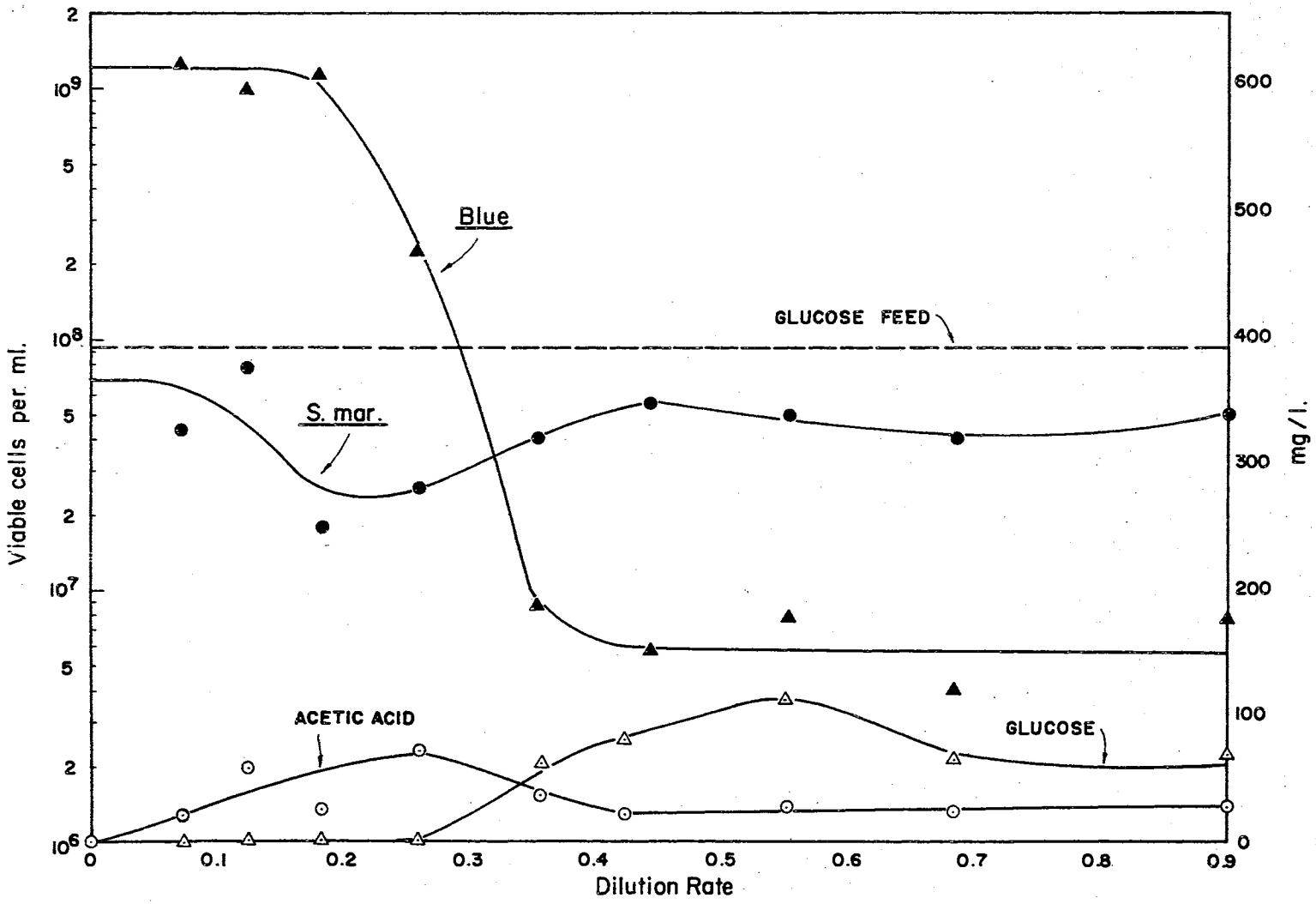

Figure 93. Experimental dilute-out curves for Serratia marcescens and Blue organism in a mixed system.

that a dense growth of Serratia marcescens had become attached to the walls and bottom. The concentration of glucose in the effluent was very low, as can be seen from the glucose curve in Figure 93. Acetic acid analyses showed that the maximum amount of intermediates was produced during the period when Blue organism was still in the system.

Figure 94 shows the results of an experiment with Pseudomonas aeruginosa and Blue organism; magnetic stirring was employed. Both organisms diluted out well, and most of the glucose appeared in the effluent at high dilution rates. No volatile acid analyses were performed in connection with this experiment.

Analysis

The Serratia marcescens-Blue organism system (Figure 93) behaved in a very peculiar way in that no dilute-out effect was observed with Serratia marcescens. It appears that this behavior can be explained by the heavy growth of the organism on the walls of the vessel. Such attachment, which was also observed to a lesser extent in the pure culture experiment with this organism (Figure 84), indicates that Serratia marcescens may not be a very satisfactory species to work with under these conditions.

It is obvious that in cases such as this, methods must be devised to maintain the cells in a dispersed state if predominance predictions based on μ_{max} are to be valid. It is very possible that in such experiments the predominating organism at high dilution rates would be the one

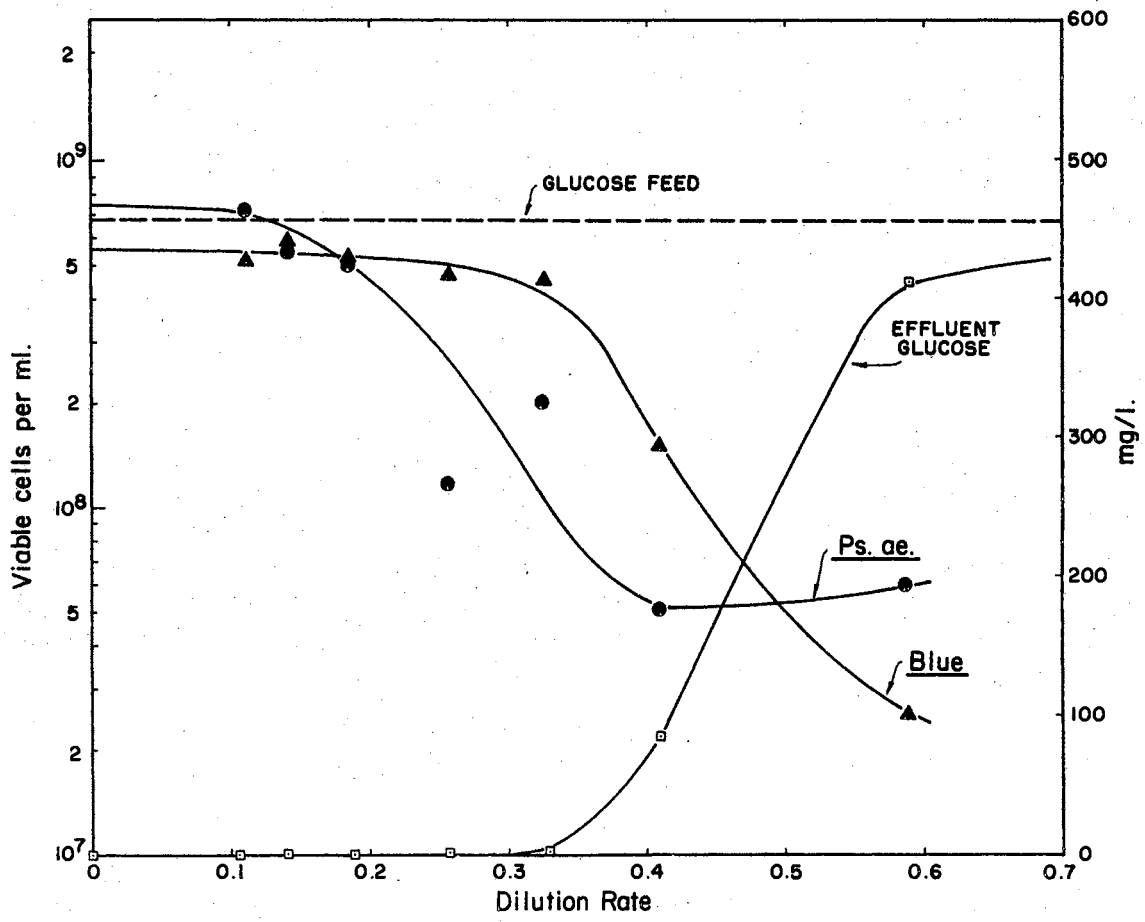

Figure 94. Experimental dilute-out curves for Blue organism and Pseudomonas aeruginosa in a mixed system with magnetic stirrer assistance.

capable of covering the inner vessel surfaces rather than those growing at higher rates. This effect also demonstrates a method whereby one organism can gain a competitive advantage over another in a natural ecological system. In the waste water field this phenomenon plays a very important role in trickling filters, and possibly also in activated sludge units.

Predictions of cell distribution at low dilution rates based on the kinetic growth rate constants already computed (Tables II and III) would indicate that Blue organism should predominate over Serratia marcescens. As can be seen from Figure 93, Blue organism was more numerous than Serratia marcescens. It is difficult to draw valid conclusions from further predominance predictions for the experiments, since Serratia marcescens, which should have diluted out first, did not do so for the reasons stated above.

Volatile acid analyses for the Serratia marcescens-Blue organism experiment indicated that acetic acid was present in measurable amounts. It is apparent from Figure 93 that the major amount of acetic acid being produced was due to Blue organism.

An examination of the kinetic constants (Table IV) shows that K_s values for Pseudomonas aeruginosa and Blue organism (Figure 94) were very similar, while their μ_{max} values differed somewhat (0.331 and 0.387, respectively). Based on these values, the prediction of predominance for the combined system would be that Blue organism might be

expected to predominate at low D values and Pseudomonas aeruginosa should dilute out slightly faster. An examination of Figure 94 shows that, as expected, Pseudomonas aeruginosa did begin to dilute out more rapidly, but at low dilution rates, i.e., 0.1, it was slightly more numerous than Blue organism.

2. Shock Load Response with Two or More Pure Cultures in a Mixed System

These experiments were carried out by incubating the organisms in a chemostat under steady state, continuous flow conditions and imposing on the system various qualitative and quantitative shock loads. Figure 95 shows the results of an experiment with Serratia marcescens and Pseudomonas aeruginosa at a constant dilution rate of 0.150. A steady state condition was obtained at the glucose level of 500 mg/l before change in the feed. The viable count showed a higher concentration of Pseudomonas aeruginosa than Serratia marcescens. The system was then subjected to a quantitative shock load by increasing the glucose concentration in the feed to 1000 mg/l. The system responded initially by showing an increase in the numbers of Serratia marcescens while retaining the same population level of Pseudomonas aeruginosa. After a period of time, the numbers of the latter organism were seen to rise.

Although no glucose was detected in the effluent, as a result of the shock, a small amount of acetic acid was produced.

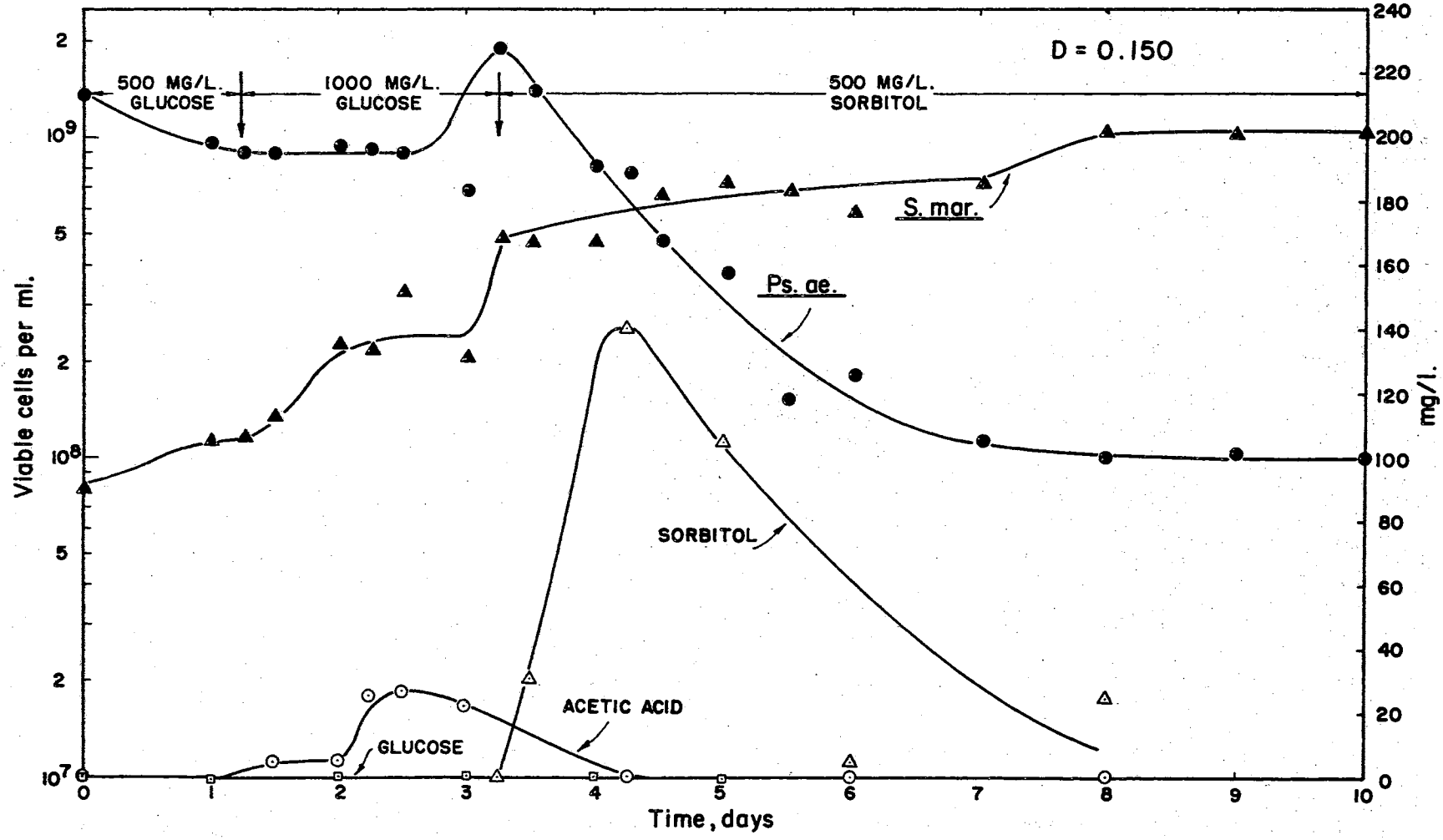

Figure 95. Shock loading response of Serratia marcescens and Pseudomonas aeruginosa in a continuous flow system.

The last shock to the system consisted of a change in the carbon source to 500 mg/l sorbitol. The system showed a temporary increase in the sorbitol concentration of the effluent, while the acetic acid concentration decreased to zero. Serratia marcescens showed an increase in density under the new conditions while Pseudomonas aeruginosa responded with a decrease in its viable numbers.

Results of an experiment using Escherichia intermedia and Serratia marcescens are presented in Figure 96. An initial dilution rate of 0.166 was used, and the feed concentration was 500 mg/l glucose. After a steady state had been approximated, the substrate concentration of the feed was changed to 1000 mg/l glucose. The viable numbers of both organisms increased, the relative increase in Serratia marcescens (which doubled nearly five times), being proportionally larger than that of Escherichia intermedia (which doubled approximately once). A small amount of acetic acid was detected in the effluent under the shock conditions, although no glucose was found. A subsequent decrease in the glucose feed to 500 mg/l brought about a return to approximately the same conditions in the system that were observed during the first phase of the experiment. A qualitative shock load of 500 mg/l sucrose resulted in a small decrease in the numbers of Serratia marcescens followed by a recovery, while imposition of a hydraulic shock load (D = 0.285) produced a decrease in the number of Escherichia intermedia, followed by a recovery to its previous

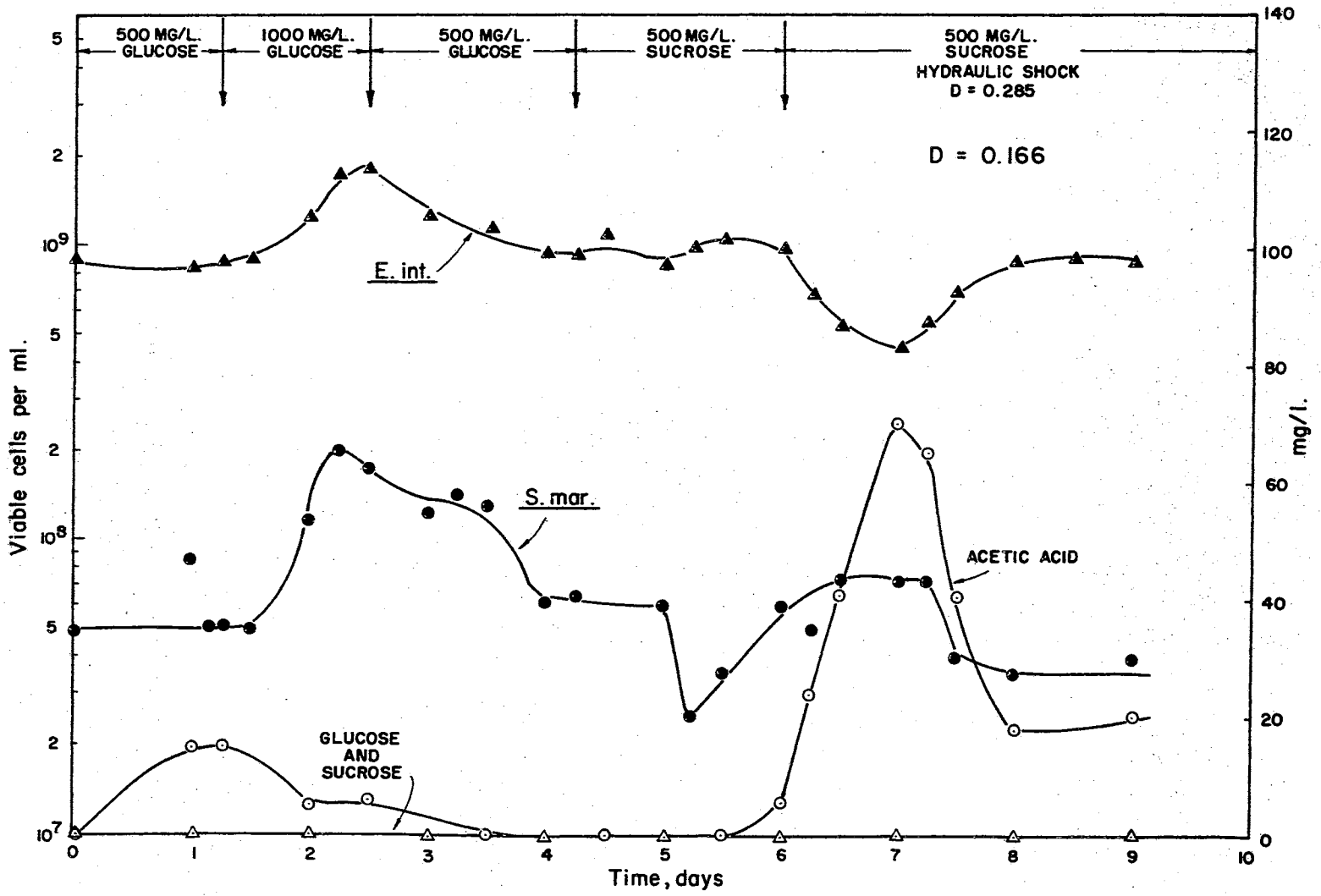

Figure 96. Shock loading response of Serratia marcescens and Escherichia intermedia in a continuous flow system.

population density. Some decrease in the numbers of the other organism was noted.

An increase in the acetic acid concentration in the effluent coincided with the observed decrease in viable numbers of Escherichia intermedia.

In Figure 97 results of an experiment with Pseudomonas aeruginosa and Blue organism, using a dilution ratio of 0.143, are presented. After a steady state had been established, a quantitative shock load (500 mg/l to 1000 mg/l glucose) brought about the establishment of higher population levels. A decrease in glucose to 500 mg/l resulted in a decrease in Pseudomonas aeruginosa numbers coupled with a numerical increase in the Blue organism viable count. To a qualitative shock (500 mg/l sorbitol) the system responded by a further increase in Blue organism and a small decrease in Pseudomonas aeruginosa. Acetic acid was noted during the first and second phases of the experiment, and it was seen to approach zero after the substrate feed was reduced to 500 mg/l glucose.

In Figure 98 results of an experiment using Pseudomonas aeruginosa, Blue organism, and Serratia marcescens are presented. The dilution rate used was 0.201. Pseudomonas aeruginosa responded to the quantitative shock loads by maintaining its population density at approximately the same level, and to the sorbitol shock by a reduction in viable numbers.

Blue organism showed an increase in numbers following

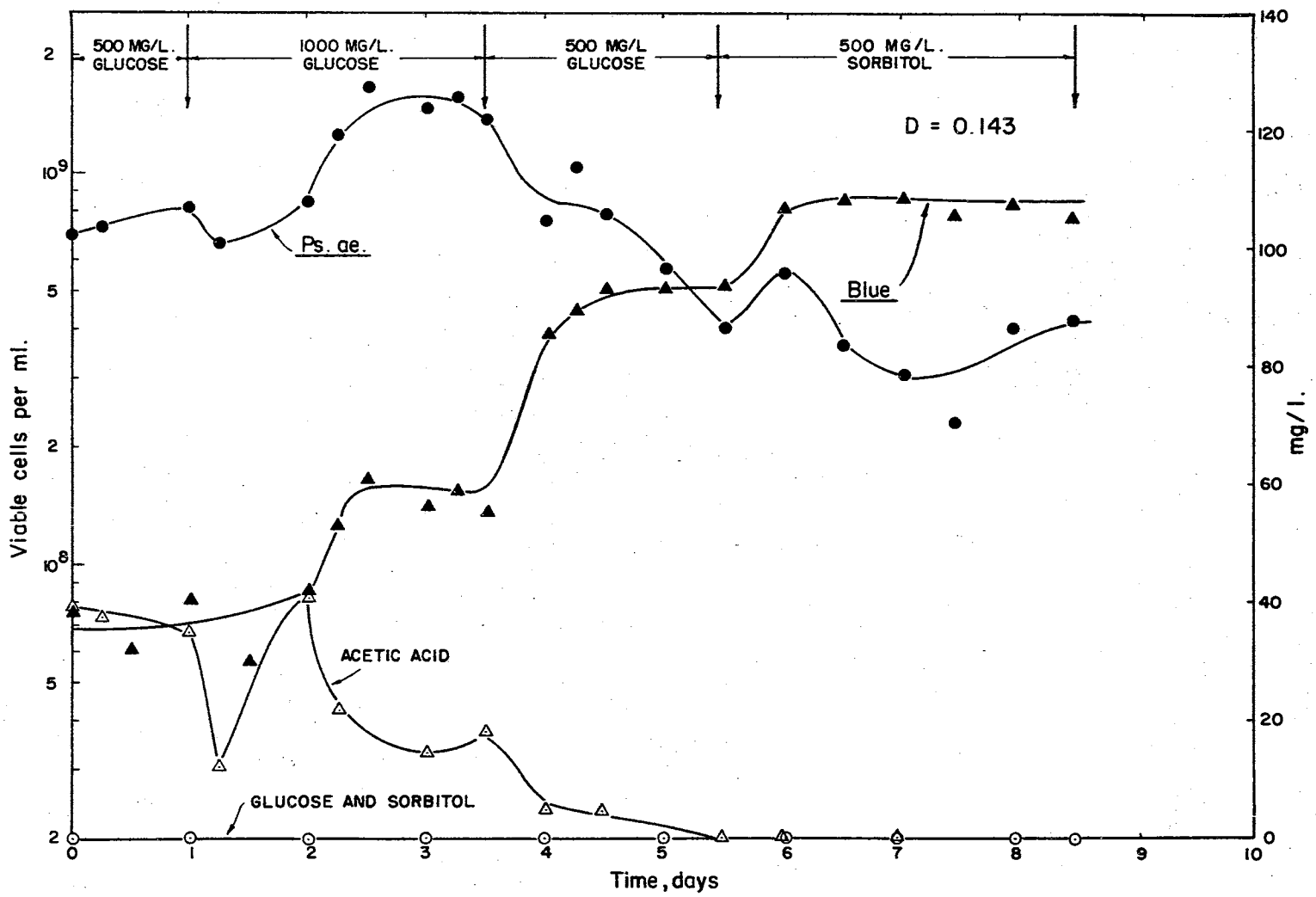

Figure 97. Shock loading response of Blue organism and Pseudomonas aeruginosa in a continuous flow system.

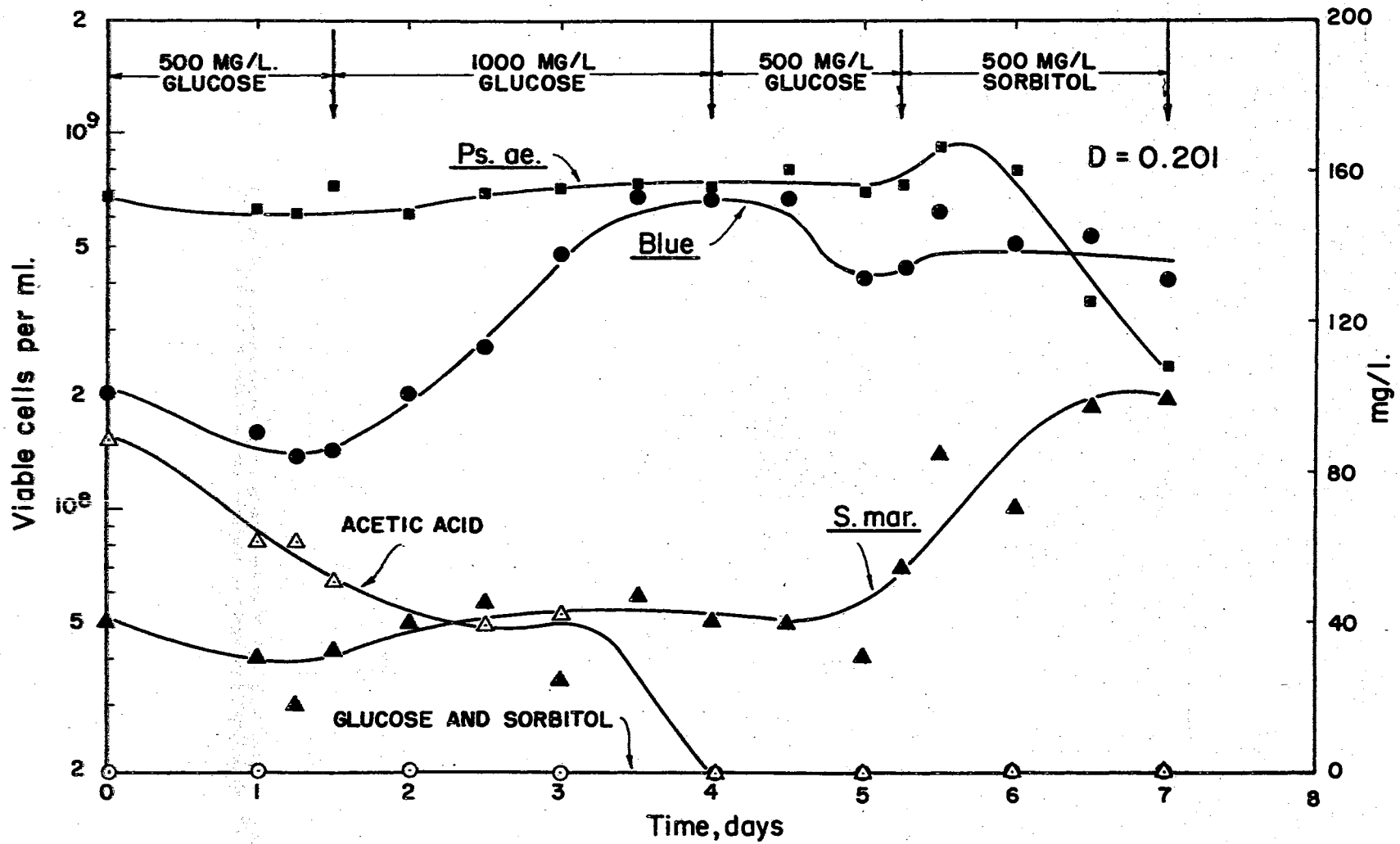

Figure 98. Shock loading response of Blue organism, Serrati marcescens, and Pseudomonas aeruginosa in a continuous flow system.

the 1000 mg/l glucose shock and a decrease in viable numbers after the feed had been returned to 500 mg/l glucose. It remained at this latter level after sorbitol was substituted as the carbon source. Serratia marcescens did not show a significant increase in viable numbers until after sorbitol was applied. Neither sorbitol nor glucose was detected in the effluent, and acetic acid was found only during the first two phases of the experiment.

Analysis

In the initial steady state portion of the two-organism experiments (Figures 95-97) predictions for the relative distribution of the bacterial species based on the batch kinetic constants were correct in two out of three cases. For example, in the experiment using Pseudomonas aeruginosa and Serratia marcescens (Figure 95) an examination of the respective μ_{max} values indicated that under steady state conditions Pseudomonas aeruginosa would predominate over the other organism. This was also observed with Escherichia intermedia and Serratia marcescens (Figure 96). It may be concluded that when two organisms are placed together under steady state conditions, the predominating organism can be predicted with a reasonable degree of certainty, particularly if the μ_{max} values for the two organisms concerned are quite different, and if no antagonistic relationships are present. However, the results shown in Figure 98 could not be predicted by consideration of μ_{max} values. In this experiment, using Pseudomonas aeruginosa, Serratia

marcescens and Blue organism, the prediction that Blue organism would predominate, followed closely by Pseudomonas aeruginosa with lesser numbers of Serratia marcescens was not confirmed. It seems apparent that, under these experimental conditions, there was a more complex interrelationship between the organisms than was predicted by simple substrate competition based on μ_{max} values. Furthermore, Figure 97 shows that predictions based on μ_{max} did not hold valid in the case of Pseudomonas aeruginosa and Blue organism. In this case, after the initial steady state had been obtained, Pseudomonas aeruginosa was shown to be the predominating organism, and it remained so through the 1000 mg/l quantitative shock even though its μ_{max} was lower.

It now becomes apparent that in some of these experiments a particular interaction between certain organisms takes place that cannot be explained on the basis of substrate competition. This unexplained interaction occurred only in cases which involved the organism Pseudomonas aeruginosa. An antagonistic effect from Pseudomonas aeruginosa is, therefore, strongly suspected.

From Figure 97 it can be noted that different population levels for Blue organism and Pseudomonas aeruginosa were present during the two periods where steady state conditions were established at 500 mg/l glucose. Such behavior may also be seen in Figure 98 with Blue organism. This phenomenon cannot be explained by kinetic theory, and

therefore it seems likely that the effect is a reflection of the antagonistic effect assigned to Pseudomonas aeruginosa.

In the case of Escherichia intermedia and Serratia marcescens shown in Figure 96 and in the case of Blue organism and Pseudomonas aeruginosa shown in Figure 97, the system response to the shock load from 500 to 1000 mg/l glucose was the establishment of new steady states in which both cultures showed a higher cell density. In Figure 97, however, a decrease in glucose feed to 500 mg/l concentration did not result in a return to the previously noted levels of cell density but caused a further decrease in Pseudomonas aeruginosa numbers and an increase in Blue organism. In fact, this second steady state in Figure 97 resulted in producing population densities that match predictions made based upon kinetic constant values. This behavior is most surprising, and it is interesting to postulate that the deleterious effect of Pseudomonas aeruginosa was somehow removed to a large degree after the second shock load, with the resulting conditions now being similar to those observed in Figure 94 at the faster dilution rates. It seems possible that if one organism exhibits an antagonistic effect over another, it may do so by elaborating some substance which is produced in greater or lesser amount, depending upon the rate at which it is growing.

An examination of Figure 95 shows that after the first shock load (1000 mg/l glucose) the population level of

Pseudomonas aeruginosa remained static until just prior to the next shock while the number of Serratia marcescens showed an increase. The same phenomenon is observed in Figure 98 where, in response to a quantitative shock load, the numbers of Serratia marcescens and Blue organism increased while Pseudomonas aeruginosa remained static. Although such behavior can be attributed to special characteristics of Pseudomonas aeruginosa, the possibility of establishing different relative population densities as a result of changes in substrate concentration is evident.

In analyzing the acetic acid concentration in these experiments, it may be observed that the method utilized to test for steady state in a continuous flow system, e.g., steady optical density for at least three detention times, may not be sufficiently accurate. In Figure 98 the acetic acid curve during the first 500 mg/l glucose feeding period definitely shows that a complete steady state situation had not been attained even though all other measurements showed no significant change. Gas chromatographic acetic acid checks, which can be performed within a very short time, should give definite confirmation of a steady state condition.

The determination of acetic acid values provided an interesting insight into the biochemical behavior of these systems when shock loaded. It can be seen that in response to the quantitative shock load the level of intermediates in the system increased, followed by a decrease until steady

state was established (Figures 95 and 97). Acetic acid also appeared in the effluent after qualitative and hydraulic shock loads, as can be observed from Figure 96. In general, it may be said from the effluent analyses performed that the continuous flow systems developed could absorb the various shocks applied, but not without some deterioration and subsequent recovery of the effluent quality.

F. Species Predominance Control Experiments

In batch systems, experiments were performed by adding 25 ml of a culture of Blue organism to a sewage seed placed in 1000 mg/l glucose, using a three-liter open vessel aerated by means of diffusers. After twenty-four hours the culture had turned a bluish color characteristic of the Blue organism. A streak of the mixture revealed what appeared to be a pure culture of Blue organism. The same situation was maintained for forty-eight additional hours through two 24-hour feeding cycles, after which the mixed liquor turned brown in color, and streaks on nutrient agar revealed that the test organism was no longer the predominating species.

In an attempt to effect a predominance change in batch systems once a natural heterogeneous population had been established, 25 ml of a culture of Blue organism were added to the batch system during one of the regular waste-and-feed periods. Although the experiment was repeated several times, no changes in predominance were observed as

result of the culture addition.

In an attempt to induce a change in predominance with a continuous flow system, one of the regular chemostats used in the Bioengineering laboratories was chosen. The system was operating on 500 mg/l glucose under a steady state condition. A heterogeneous microbial population was present, the system having been developed from sewage seed. Every twelve hours 5 ml of a heavy suspension of Serratia marcescens were introduced directly into the aerating liquor by means of a pipette. This procedure was followed for seven days. For another week after the above procedure was followed, 4 ml of Serratia marcescens suspension were added four times a day. During the entire experimental run frequent plate counts were made. No change in the physical appearance of the aerating liquor was noted, and the plate counts failed to reveal the presence of Serratia marcescens cells.

Analysis

The experiments reported in this section were of a very exploratory nature, and it is not felt that any conclusions may be derived from them. It may be noted, however, that in a batch system a short-term predominance shift was attained by adding a massive inoculum of an organism with a high μ_{max} to the original sewage seed. In similar batch systems it was not possible to induce a predominance change after the original seed had developed an established bacterial population. It is interesting to

note that even after a large population of Blue organism had been established by massive inoculations, it did not maintain its predominance for more than forty-eight hours. Such results would indicate the difficulties involved, not only in inducing predominance changes, but also in maintaining the population selected for a period of time.

In continuous flow units, none of the predominance changes attempted was successful. It would appear that the competitive situations in a steady state system are less susceptible to predominance changes induced by seed addition. It seems likely that in the open system, most of the added cells were diluted out before they had an opportunity to become established in the aerating liquor.

In the batch systems, a blue color identified the presence of Blue organism. The use of color patterns to detect massive predominance changes has been suggested by Rao and Gaudy (95), and Cassell, Sulzer, and Lamb (99). This method, however, should be viewed with caution, because other factors (which do not necessarily involve predominance changes) influence color in bacterial cultures. For example, the organism herein termed "Blue organism" because it imparts a bluish tint to the mixed liquor, does so only during an active growth phase.

CHAPTER VI

DISCUSSION

In this section it appears desirable to mention the most common problems encountered in the actual execution of the experiments. The paramount difficulty in carrying out experiments involving pure culture systems was maintaining the purity of the species by avoiding contamination. It is necessary to emphasize that the greatest care must be taken in every step of the experiments to ensure that no contamination is introduced. The smallest deviation from good sterile techniques may result in the loss of one or more experiments because of contamination difficulties. It is to be noted that the usual method of determining purity is by streaks or plate counts, and these tests take a certain amount of time to develop, thus in many cases making it impossible to determine if cultures remain pure until after an experimental run has been completed. Difficulty was experienced in sterilizing the media and the equipment, and keeping the system uncontaminated in continuous flow runs, possibly because of the long time involved in each run. It is in connection with continuous flow experiments that care in employing pure culture techniques should be maximized.

The experiments herein reported, batch as well as continuous flow, were devoid of detectible contamination. There were times during the work when contamination was detected. In all of these cases the experiments were discarded and re-run.

The use of the kinetic growth rate constants in the prediction of predominance patterns in batch and continuous flow systems are two of the subjects upon which this report is intended to cast some light. In batch systems it was found possible to predict the predominating organism when two or three species of bacteria were incubated together, based on μ_{max} values. Such predictions, however, must be qualified by several conditions, as follows:

1. It is necessary that no organism exhibit a significant antagonistic effect on the other culture or cultures during growth. Such antagonism would not permit a predominance based on competition for the available substrate. Cases of antagonistic effects have been noted in Figures 67 and 71.
2. It is necessary that no significant growth log occur with one of the cultures. Such a case is illustrated by Pseudomonas aeruginosa, where a lag of over two hours was observed for the organism (Figure 77).
3. It is desirable that the initial bacterial seed consist of approximately the same number of each organism. It is possible that in a case

where a disproportionate number of cells in one of the bacterial seeds exists, the qualitative predominance prediction may not be correct, although it is to be noted that the ratio of the viable numbers for both species will change through the growth period. An example of this effect is shown in Figure 65 with Yellow organism and Escherichia intermedia.

In view of the results obtained from batch systems, it may be surmised that the possibility exists that prediction of predominance patterns for systems containing more than three organisms may be accomplished by following the same procedures as presented in this report.

From several continuous flow experiments in which no antagonistic relationship appeared to take place, it was found to be possible to predict predominance patterns based on an evaluation of μ_{max} values for each of the organisms. Figures 93, 94, 95, and 96 show that such predictions are possible. Certain limitations and qualifications for such predictions must be stated. These are:

1. Sufficient agitation should be maintained in the aeration vessel to prevent excessive attachment of cells to the reactor walls and bottom, or such growth must be removed by other means without altering the existing steady state conditions.

2. Predictions may not be made for systems containing more than two organisms. It is very possible that such predictions could be made, but no evidence for such an extension is presented in this report. The only triple continuous flow experiment reported (Figure 98) was so strongly influenced by antagonistic relationships that it could not be used as a basis for surmising further possibilities of predicting predominance.

The evidence collected in this work points to the possible importance of using μ_{max} values of organisms obtained in pure culture studies to predict predominance in mixed systems. The K_s for the organisms was found, in the batch runs, to be of little significance in the prediction of predominance in most cases examined. However, the high K_s value for Yellow organism could explain its restrained growth or decrease in some mixed experiments (see Figures 65, 67, 71, and 78). It is possible that K_s could play an important role in predominance patterns at substrate levels substantially lower than those utilized in this work. It is also possible that K_s values could be of importance in steady state systems, or during some transient state conditions. This latter possibility, however, could not be evaluated in the present study, since the difference in K_s for the organisms employed in continuous flow experiments was not great.

It was anticipated that the elaboration of metabolic intermediates derived from glucose metabolism would have an effect on the determination of predominance patterns. An example of such an effect may be noted in the growth of Escherichia intermedia. In the mixed systems including this organism, it may be seen that no secondary oxygen uptake took place as was noted in the pure culture. It can be surmised that other organisms in a mixed culture were able to metabolize the intermediates excreted by Escherichia intermedia, thus benefiting from the association, e.g., Figures 56 and 62. It is also possible that the lack of growth noted with Yellow organism (which did not accumulate intermediates during growth on glucose) when in a mixed culture with Blue organism and Serratia marcescens (Figures 67 and 71) may be due to some metabolic intermediate or end product introduced into the medium by the latter organisms.

Gas chromatographic analyses showed that acetic acid was produced as a metabolic intermediate of glucose utilization in all cases tested. No other volatile acid was detected. On the other hand, metabolism of sorbitol yielded no acetic acid at all, thus pointing to the possibility that glucose and sorbitol are metabolized by different pathways. In the case of Escherichia intermedia, the intermediates produced did not appear to be different from those produced by the other organisms; therefore, it seems that acetic acid is produced in a majority of cases, and that Escherichia intermedia is not capable of utilizing this acid

immediately without a period of acclimation or synthesis of new enzymes. This organism is apparently one of the few that requires a lengthy acclimation process in order to metabolize acetic acid. In this study, glucose and the intermediates produced were always metabolized sequentially; however, this was reflected as a plateau in the oxygen uptake curve, COD removal curve, etc., only in the case of Escherichia intermedia. A similar plateau effect caused by the need for an acclimation period in the two-substrate systems has been shown in several of the growth rate comparison experiments.

The case of Pseudomonas aeruginosa is a very interesting one. As shown in several of the batch mixed culture runs, no antagonistic relationships were noted when this organism was combined with other species. However, in continuous flow experiments (Figures 97 and 98) there was a depressing effect on the numbers of other organisms grown in association with Pseudomonas aeruginosa. In addition, the endogenous experiments presented in Figure 84 show a decrease in the number of Serratia marcescens, while Pseudomonas aeruginosa increased in density. Another unusual response of this organism is that it grows in the presence of sorbitol when grown in association with Serratia marcescens, although it cannot metabolize sorbitol by itself.

From the observations on Pseudomonas aeruginosa in combination with other species, it may be surmised that this

organism benefits, under certain conditions, from its association with other cells. The beneficial effect may not necessarily be one conferred on Pseudomonas aeruginosa by the other organisms, but may be due to a detrimental effect of Pseudomonas aeruginosa on the others. This detrimental effect may involve a product excreted by Pseudomonas aeruginosa. It seems possible that the soluble pigment produced by Pseudomonas aeruginosa is an agent causing the relationships noted. If such a product or pigment is able to induce lysis, or to retard growth of other cells, the effects observed would be explained. In continuous flow experiments, the substance could decrease growth of other strains, resulting in higher relative numbers of Pseudomonas aeruginosa. In the endogenous runs, the organism could benefit from products of cell lysis from the other species. It also seems possible that a substance excreted by Pseudomonas aeruginosa could accelerate lysis or reduce the uptake by the other intact cells of carbon made available by lysis.

In the case of the mixed culture experiments using sorbitol as a carbon source, where Pseudomonas aeruginosa grew in the presence of Serratia marcescens, the effect may have taken place as a result of intermediates produced by the latter organism. Another possibility could be that a substance excreted by Pseudomonas aeruginosa could induce lysis in some Serratia marcescens cells, with the cell lysis products being assimilated by Pseudomonas aeruginosa.

A third, but remote, possibility could be that Serratia marcescens elaborated some product which permitted Pseudomonas aeruginosa to induce the necessary enzyme(s) to grow on sorbitol.

The foregoing discussion concerning the behavior of Pseudomonas aeruginosa in combination with other organisms is highly speculative, but it is felt that many of the effects noted may be related to the soluble pigment produced by this organism. This aspect could be investigated by studying the effect of the soluble pigment on the growth and metabolic patterns of the organisms which were used in association with Pseudomonas aeruginosa. Such investigations were not undertaken in the present research, since the aim was to gain a broad insight into predominance patterns. It is realized, however, that the Pseudomonas aeruginosa mixtures could provide extremely valuable systems for more penetrating study, especially if continued research indicates that other soluble pigment producers exert a suppressing influence on the growth of a wide variety of other organisms. The effect in itself is worthy of study from a basic standpoint. However, in order to justify study from an engineering standpoing, the phenomenon would have to be exhibited by other organisms as well as Pseudomonas aeruginosa.

In analyzing the results of the dilute-out experiments with single organisms in comparison with the theoretical values predicted using μ_{max} and K_s determined from batch

experiments, it was noted that a close fit was never realized. Even in those experiments where magnetic stirring was utilized (Figures 91 and 92), the observed and theoretical curves did not coincide. There was, however, a similarity in the shape and trend of the curves. The observed substrate values were seen to be generally displaced to the right along the dilution rate axes. The observed cell concentrations were higher than those of the theoretical curves (see Figure 92). It is perhaps proper to theorize that the behavior of a system such as the one shown in Figure 92 is due either to practical experimental deficiencies in the equipment, or to specific properties of the bacteria that do not produce a completely dispersed system in the reactor. It could be that both aspects are interrelated, and that better results could be obtained by the utilization of a larger chemostat provided with several air diffusers to increase mixing. These observations underline the practical difficulties in dealing with living bacterial systems, and suggest that a clearer understanding of the limitations of the theory when applied to practice is needed in order that results may be analyzed in a more meaningful manner.

The shock load response of mixed systems indicates that a change in predominance may be obtained with a qualitative shock load, as in the case of Pseudomonas aeruginosa and Serratia marcescens when placed on sorbitol (Figure 95). Also interesting is the observation that it is possible to

change the relative composition of the population by means of a quantitative shock load (Figure 97).

The present work was necessarily of an exploratory nature, since this area of investigation is extremely broad and very diffuse. Also, although considerable work on species predominance has been accomplished in some basic and applied microbiological areas, the amount of work accomplished is small in relation to the ultimate importance of this area. Much of the work which has been reported is of little direct use in the water pollution control field. While the present study has admittedly only "scratched the surface," it has shown that gross estimates of species predominance do appear to be attainable using closely controlled model systems under experimental conditions (temperature, substrate levels, dilution rates, etc.), approaching those present in biological treatment facilities, and it is possible to draw the tentative conclusions given in the following chapter.

CHAPTER VII

CONCLUSIONS

1. The values of μ_{max} for pure cultures can be used to predict predominance patterns in a qualitative sense in batch and continuous flow systems of two or three individual organisms when no antagonistic interactions are evident in the system

2. No significant role in the prediction of predominance patterns was evident for the constant K_s at the substrate levels utilized.

3. The growth plateau observed using a pure culture system with a two-substrate carbon source appears to be the result of sequential substrate removal and may or may not be masked or enlarged by acclimation of the seed.

4. The work with Escherichia intermedia confirms the work of Bhatla and Gaudy (91) on the causes of the growth plateau observed with pure cultures.

5. The sequential substrate removal in a glucose-sorbitol system observed by Gaudy, Komolrit, and Bhatla (109) for heterogeneous populations has been substantiated by similar behavior observed with a variety of pure cultures.

6. Acetic acid comprised the bulk of the volatile acid intermediates accumulated during glucose metabolism by the bacterial cultures examined.

7. An interrelationship between Pseudomonas aeruginosa and other organisms has been detected. Such a relationship is beneficial to the growth of Pseudomonas aeruginosa in the presence or absence of exogenous substrate. It is suggested that the soluble pigment produced by Pseudomonas aeruginosa may play a significant role in the establishment of this effect.

8. The curves derived from the theoretical formulations for steady state kinetics using constants determined in batch studies were found to be similar to the observed curves in shape and trend but not in detail, particularly at high dilution rates.

9. Changes in predominance or in relative numbers of bacterial species present in continuous flow systems can be brought about in response to quantitative, qualitative, and hydraulic shock loadings.

CHAPTER VIII

SUGGESTIONS FOR FUTURE WORK

It is felt that the following research ideas would be of value in undertaking future investigations:

1. Since the constant K_s may play an important role in predominance predictions at low substrate levels, it appears important to study such an effect.

2. In order to ascertain if quantitative predominance predictions may be made based on bacterial kinetic growth rate constants, it is suggested that additional work be performed using simple model batch systems of two or three organisms.

3. It appears desirable to study more complex mixed culture systems involving several bacterial species to determine if gross predominance predictions may be made using such systems.

4. In order to gain additional information on the behavior of mixed bacterial systems when subjected to quantitative, qualitative and hydraulic shock loads, it is felt that additional continuous flow work in this area would be of considerable interest.

5. An interrelationship between Pseudomonas aeruginosa and other organisms, particularly Serratia marcescens, has been detected in this work. It would be of interest to continue research into the causes and results of this effect.

6. It is suggested that exploratory work be undertaken into the influence of bacteriophage action upon mixed bacterial systems. In the water pollution control field, results of this type of work could be of considerable interest in determining the role of bacterial viruses in the selection of natural bacterial populations.

SELECTED BIBLIOGRAPHY

1. Waksman, S. A., "Associative and Antagonistic Effects of Microorganisms. I. Historical Review of Antagonistic Relationships." Soil Science, 43, 51-68 (1937).

2. Hall, I. C., and E. Peterson, "The Effects of Certain Bacteria upon the Toxin Production of Bacillus botulinus in Vitro." J. Bacteriology, 8, 319-341 (1923).

3. Sherman, J. M., and R. H. Shaw, "Associative Bacterial Action in the Propionic Acid Fermentation." J. Gen. Physiology, 3, 657-658 (1921).

4. Sturges, W. S., and L. F. Pettger, "Methods for the Isolation and Cultivation of Bacillus putrificus and Other Obligate Anaerobes." J. Bacteriology, 4, 171-175 (1919).

5. Hutchinson, H. B., and J. Clayton, "On the Decomposition of Cellulose by an Aerobic Organism." J. Agricultural Science, 9, 1943-173 (1919).

6. Sanborn, J. R., "Physiological Studies of Association." J. Bacteriology, 12, 343-353 (1926).

7. Broom, J. C., "The Exhaustion of Media in Bacterial Culture." Brit. J. Exp. Path. 10, 71-83 (1929).

8. Waksman, S. A., and J. W. Foster, "Associative and Antagonistic Effects of Microorganisms. II. Antagonistic Effects of Microorganisms Grown on Artificial Substrates." Soil Science, 43, 69-76 (1937).

9. Holman, W. L., and D. M. Meekison, "Gas Production by Bacterial Synergism." J. Infect. Diseases, 39, 145-172 (1926).

10. Lederberg, J., "Studies in Bacterial Genetics." J. Bacteriology, 52, 503 (1946).

11. Rahn, O., "Mutual Influences." Marshall's Microbiology. P. Blakinston's Sons and Co., Philadelphia (1921).

12. Fawcett, H. S., "The Importance of Investigations on the Effects of Known Mixtures of Microorganisms." Phytopathology, 21, 545-550 (1931).

13. Savastano, G., and H. S. Fawcett, "A Study of Decay in Citrus Fruits Produced by Inoculations with Known Mixtures of Fungi at Different Constant Temperatures." J. Agricultural Research, 39, 163-198 (1929).

14. Waksman, S. A., and I. J. Hutchings, "Associative Antagonistic Effects of Microorganism. III. Associative and Antagonistic Relationships in the Decomposition of Plant Residues." Soil Science, 43, 77-92 (1937).

15. Speakman, H. B., and J. F. Phillips, "A Study of a Bacterial Association. I. The Biochemistry of the Production of Lactic Acid." J. Bacteriol., 9, 183-200 (1924).

16. Waksman, S. A., and S. Lomanits, "Contribution to the Chemistry of Decomposition of Proteins and Amino Acids by Various Groups of Microorganisms." J. Agricultural Research, 30, 263-281 (1925).

17. Buchanan, R. E., and E. I. Fulmer, Physiology and Biology of Bacteria, Vol. III. The Williams and Wilkins Company, Baltimore, Maryland (1930).

18. Fulton, M., "Antibiosis in the Colon-Typhoid Group. I. Growth Curves of Two Strains in a Synthetic Medium." J. Bacteriology, 34, 301-315 (1937).

19. Kesel, R. G., "The Biological Production and Therapeutic Use of Ammonia in the Oral Cavity in Relation to Dental Caries." J. Am. Dental Association, 33, 695-714 (1946).

20. Jeney, A. "Antibiotic Effects of d-Amino Acids." Hungarica Acta Physiol., 1, 142-145 (1948). From Biological Abstracts, 22, 21395 (1948).

21. Charlton, G., "Direct Antagonism in Mixed Bacterial Populations." J. Bacteriology, 70, 56-59 (1955).

22. Wynne, E. S., "Antagonism by Aerobacter Strains." J. Bacteriology, 53, 469-478 (1947).

23. Frankel, J. J., and E. S. Wynne, "Antagonism by Gaffkya tetragena Strains." J. Infect. Diseases, 89, 52-55 (1951).

24. Bowling, R. E., and E. S. Wynne, "Studies on the Mechanism of Antagonism by Aerobacter Strains." J. Infect. Diseases, 89, 277-280 (1951).

25. Wynne, E. S., and J. O. Norman, "On the Concept of Direct Antagonism in Bacteria." J. Infect. Diseases, 93, 243-246 (1953).

26. Lockhart, W. R., and D. M. Powelson, "Staling in Bacterial Culturing." J. Bacteriology, 65, 293-296 (1953).

27. Borgstrom, G., "Microbiological Problems of Frozen Food Products." Advances in Food Research, 6, 163-230 (1955).

28. Peterson, A. C., J. J. Black, and M. F. Gunderson, Staphylococci in Competition. I. Growth of Naturally-occurring Mixed Populations in Precooked Frozen Foods during Defrost." Applied Microbiology, 10, 16-22 (1962).

29. Peterson, A. C., J. J. Black, and M. F. Gunderson, "Staphylococci in Competition. II. Effect of Total Numbers and Proportion of Staphylococci in Mixed Cultures on Growth in Artificial Culture Medium." Applied Microbiology, 10, 23-30 (1962).

30. Peterson, A. C., J. J. Black, and M. F. Gunderson, "Staphylococci in Competition. III. Influence of Salt on Staphyloccal Growth in Mixed Populations." Applied Microbiology, 12, 70-76 (1964).

31. Peterson, A. C., J. J. Black, and M. F. Gunderson, "Staphylococci in Competition. IV. Effect of Starch and Kind and Concentration of Sugar on Staphylococcal Growth in Mixed Populations." Applied Microbiology, 12, 77-82 (1964).

32. Flippin, R. S., and M. N. Mickelson, "Use of Salmonellae Antagonists in Fermenting Egg White. I. Microbial Antagonists of Salmonellae. Applied Microbiology, 8, 366-370 (1960).

33. Mickelson, M. N., and R. S. Flippin, "Use of Salmonellae Antagonists in Fermenting Egg White. II. Microbiological Methods for the Elimination of Salmonellae from Egg White." Applied Microbiology, 8, 371-377 (1960).

34. Oberhofer, T. R., and W. C. Frazier, "Competition of Staphylococcus aureus with other Organisms." J. Milk Food Technology, 24, 172-175 (1961).

35. Iandolo, J. J., Carol W. Clark, Leslie Bluhm, and A. John Ordal, "Repression of Staphylococcus aureus in Associative Culture." Applied Microbiology, 13, 646-649 (1965).

36. Freter, R., "In Vivo and In Vitro Antagonism of Intestinal Bacteria against Shigella flexneri. II. The Inhibitory Mechanism. J. Infect. Diseases, 110, 38-46 (1962).

37. Shindala, A., Henry R. Bungay, III, Noel R. Krieg, and Kathleen Culbert, "Mixed Culture Interactions. I. Commensalism of Proteus vulgaris with Saccharomyces cerevisiae in Continuous Culture." J. Bacteriology, 89, 693-696 (1965).

38. Hentges, D. J., and M. Fulton, "Ecological Factors Influencing the Relationships between Klebsiella and Shigella in Mixed Cultures." J. Bacteriology, 87, 527-535 (1964).

39. Guthrie, R. K., B. H. Cooper, J. K. Ferguson, and H. E. Allen, "Interaction between Escherichia coli and Pseudomonas aeruginosa in Mixed Cultures." Canadian J. Microbiology, 11, 947-952 (1965).

40. Gibson, J., "Nutritional Aspects." Microbial Ecology, ed. by R. E. O. Williams and C. C. Spicer, Cambridge University Press, London (1957).

41. Rich, L. G., Unit Processes of Sanitary Engineering, John Wiley and Sons, New York (1963).

42. Pipes, W. O. "Ecological Study of Activated Sludge," Advances in Applied Microbiology, Vol. 8, Academic Press, New York (1966).

43. Buswell, A. M., "The Biology of Activated Sludge." Sewage Works Journal, 3, 362-368 (1931).

44. Winogradsky, H., "Sur la Microflore Nitrificatrice des Boues Activies de Paris." Comp. Rend. Acad. Sci. (Paris) 200, 1886-1888 (1935).

45. Hotchkiss, M., "Studies on the Biology of Sewage Disposal." New Jersey Agricultural Experiment Station Bulletin 390, 49-67 (1923).

46. Butterfield, C. T., "Studies of Sewage Purification. II. A Zoogloea-forming Bacterium Isolated from Activated Sludge." Public Health Report 50, 671-684 (1934).

47. Gilcreas, F. W., "Laboratory Control of Sewage Treatment." Municipal Sanitarion, 8, 225-227 (1937).

48. Butterfield, C. T., C. C. Ruchhoft, and P. D. McNamee, "Studies of Sewage Purification. VI. Biochemical Oxidation by Sludges Developed by Pure Culture of Bacteria Isolated from Activated Sludge." Public Health Report 52, 387-409 (1937).

49. Butterfield, C. T., and E. Wattie, "Effective Bacteria in Purification by Trickling Filters." Public Health Report 56, 2455-2464 (1941).

50. Heukelekian, H., and M. Littman, "Carbon and Nitrogen Transformations in the Purification of Sewage by the Activated Sludge Process. II. Morphological and Biochemical Studies of Zoogloeal Organisms." Sewage Works Journal, 11, 752-763 (1939).

51. Wattie, E., "Cultural Characteristics of Zoogloea-forming Bacteria Isolated from Activated Sludge and Trickling Filters." Public Health Report 57, 1519-1534 (1942).

52. Ruchhoft, C. C., "Studies of Sewage Purification. IX. Total Purification, Oxidation, Adsorption and Synthesis of Nutrient Substrate by Activated Sludge." Public Health Report 54, 468-496 (1948).

53. McKinney, R. E., and M. P. Horwood, "Fundamental Approach to the Activated Sludge Process. I. Floc-forming Bacteria." Sewage and Industrial Wastes, 24, 117-123 (1952).

54. McKinney, R. E., and R. G. Weichlein, "Isolation of Floc-producing Bacteria from Activated Sludge." Applied Microbiology, 1, 259-261 (1953).

55. Dugan, P. R., and D. G. Lundgreen, "Isolation of the Floc-forming Organism Zoogloea ramigera and Its Culture in Complex andSynthetic Media." Applied Microbiology, 8, 357-361 (1960).

56. Dias, F. F., and J. V. Bhat, "Microbial Ecology of Activated Sludge. I. Dominant Bacteria." Applied Microbiology, 12, 412-417 (1964).

57. Hawkes, H. A., The Ecology of Waste Water Treatment. The Macmillan Company, New York (1963).

58. Allen, L. A., "The Bacteriology of Activated Sludge." J. Hygiene, 43, 424-431 (1944).

59. Calway, W. T., W. R. Carrol, and S. K. Long, "Heterotrophic Bacteria Encountered in Intermittent Sand Filtration of Sewage." Sewage Industrial Wastes, 24, 642-653 (1952).

60. Hamdy, M. K., E. L. Sherrer, H. H. Weiser, and W. D. Sheets, "Microbiological Factors in the Treatment of Phenolic Wastes." Applied Microbiology, 2, 143-148 (1954).

61. Jasewicz, L., and N. Porges, "Biochemical Oxidation of Dairy Wastes. VI. Isolation and Study of Sludge Microorganisms." Sewage Industrial Wastes, 28, 1130-1136 (1956).

62. Rogovskaya, T. I., and M. F. Lazareva, "Intensification of Biochemical Purification of Industrial Sewage. I. A Microbiological Specification of Activated Sludges Purifying Various Industrial Sewages." Microbiologiya, 28, 530-538 (1959).

63. Morgan, E. H., and A. J. Beck, "Carbohydrate Wastes Stimulate Growth of Undesirable Filamentour Organisms." Sewage Works Journal, 1, 46-51 (1928).

64. Ruchhoft, C. C., and J. H. Watkins, "Bacteriological Isolation and Study of the Filamentous Organisms in the Activated Sludge of the Des Plaines River Sewage Treatment Plant." Sewage Works Journal, 1, 52-58 (1928).

65. Haseltine, T. R., "The Activated Sludge Process at Salinas, California, with Particular Reference to Causes and Control of Bulking." Sewage Works Journal, 4, 461-489 (1932).

66. Smit, J., "A Study of Conditions Favoring Bulking of Activated Sludge." Sewage Works Journal, 4, 960-972 (1932).

67. Smith, R. S., and W. C. Purdy, "Studies of Sewage Purification. IV. The Use of Chlorine for the Correction of Sludge Bulking in the Activated Sludge Process." Public Health Report 51, 617-623 (1936).

68. Ingols, R. S., and H. Heukelekian, H., "Studies on Activated Sludge Bulking. I. Bulking of Sludge by Means of Carbohydrates." Sewage Works Journal, 11, 927-945 (1939).

69. Anon., "New England Association Reviews Sewage Research." Municipal Sanitation, 10, 592-593 (1939).

70. Ruchhoft, C. C., J. F. Kachmar, and O. R. Placat, "Studies on Sewage Purification. XII. Metabolism of Glucose by Activated Sludge." Sewage Works Journal, 12, 485-503 (1940)

71. Lackey, J. B., and E. Wattie, "Studies of Sewage Purification. XIII. The Biology of Sphaerotilus natans Kutzing in relation to Bulking of Activated Sludge." Sewage Works Journal, 12, 669-684 (1940).

72. Littman, M. L., "Carbon and Nitrogen Transformations in the Purification of Sewage by the Activated Sludge Process. IV. With a Culture of Sphaerotilus." Sewage Works Journal, 12, 685-693 (1940).

73. Heukelekian, H., and R. S. Ingols, "Studies on Activated Sludge Bulking. II. Bulking Induced by Domestic Sewage." Sewage Works Journal, 12, 694-714 (1940).

74. Heukelekian, H., and R. S. Ingols, "Studies on Activated Sludge Bulking. III. Bulking of Sludge Fed with Pure Substances and Supplied with Different Amounts of Oxygen." Sewage Works Journal, 12, 849-861 (1940).

75. Bloodgood, D. E., "Discussion of Dr. Edward's Paper on New Developments in Activated Sludge Operation." Sewage and Industrial Wastes, 12, 1085-1086 (1940).

76. Ruchhoft, C. C., and J. F. Kachmar, "Studies of Sewage Purification. XIV. The Role of Sphaerotilus natans in Activated Sludge Bulking." Sewage Works Journal, 13, 3-32 (1941).

77. Logan, L. P., and W. E. Budd, "Effect of BOD Loadings on Activated Sludge Operation." Biological Treatment of Sewage and Industrial Wastes, ed. by J. McCabe and W. W. Eckenfelder. Reinhold Publishing Company, New York, 1956.

78. Heukelekian, H., and E. Weisberg, "Bound Water and Activated Sludge Bulking." Sewage and Industrial Wastes, 28, 558-574 (1956).

79. Ludzack, F. J., and R. B. Schaffer, "Temperature and Feed as Variables in Activated Sludge Performance." Journal Water Pollution Control Federation, 33, 141-156 (1961).

80. Dondero, N. C., R. A. Phillips, and H. Heukelekian, "Isolation and Preservation of Cultures of Sphaerotilus." Applied Microbiology, 9, 219-227 (1961).

81. Genetelli, E. J., and H. Heukelekian, "The Influence of Loading and Chemical Composition of Substrate on the Performance of Activated Sludge." Journal Water Pollution Control Federation, 36, 643-649 (1964).

82. Lackey, J. B., "Biology of Sewage Treatment." Sewage Works Journal, 21, 659-665 (1949).

83. Cooke, W. B., "Check List of Fungi Isolated from Polluted Water and Sewage." Syndowia Annales Mycologici., 1, 146-175 (1956).

84. Cooke, W. B., and F. J. Ludzack, "Predacious Fungus Behavior in Activated Sludge Systems." Sewage and Industrial Wastes, 30, 1490-1495 (1958).

85. Pipes, W. O., and P. H. Jones, "Decomposition of Organic Wastes by Sphaerotilus." Biotechnology and Bioengineering, V, 287-307 (1963).

86. Cooke, W. B., "Yeasts in Polluted Water and Sewage." Mycologia., 52, 210-230 (1960).

87. Purdy, W. C., and C. T. Butterfield, "The Effect of Plankton Animals upon Bacterial Death Rates." American Journal of Public Health, 8, 499-505 (1918).

88. Pillai, S. C., and V. Subrahmanyan, "Role of Protozoa in the Activated Sludge Process." Nature, 150, 525 (1942).

89. Pillai, S. C., and V. Subrahmanyan, "Role of Protozoa in the Aerobic Purification of Sewage." Nature, 154, 179-180 (1944).

90. Heukelekian, H., and M. Gurbaxani, "Effects of Certain Physical and Chemical Agents on the Bacteria and Protozoa of Activated Sludge." Sewage Works Journal, 21, 811-818 (1949).

91. Bhatla, M. N., and A. F. Gaudy, Jr., "Role of Protozoa in the Diphasis Exertion of BOD." Journal Sanitary Engineering Division, ASCE, 91, 63-87 (1965).

92. Jeris, J. S., and P. L. McCarty, "The Biochemistry of Methane Fermentation using C^{14} Tracers. Journal Water Pollution Control Federation, 37, 178-192 (1965).

93. Gaudy, A. F. Jr., "Mode of Bacterial Predominance in Aerobic Waste Disposal Systems." Master's Thesis, M.I.T. (1951).

94. Engelbrecht, R. S., and R. E. McKinney, "Activated Sludge Cultures Developed on Pure Organic Compounds." Sewage and Industrial Wastes, 29, 1350-1362 (1957).

95. Rao, B. S., and A. F. Gaudy, Jr., "Effect of Sludge Concentration on Various Aspects of Biological Activity in Activated Sludge." Journal Water Pollution Control Federation, 38, 798-812 (1966).

96. Leal, L. L. "Predominance of Bacterial Species in Steady and Non-steady State Systems." Unpublished Master's Thesis, O.S.U. (1964).

97. Kincannon, D. F., and A. F. Gaudy, Jr., "Some Effects of High Salt Concentrations on Activated Sludge." Journal Water Pollution Control Federation, 38, 1148-1159 (1966).

98. Gaudy, A. F. Jr., M. N. Bhatla, R. H. Follett, and F. Abu-Niaaj., "Factors Affecting the Existence of the Plateau during Exertion of BOD." Journal Water Pollution Control Federation, 37, 444-459 (1965).

99. Cassell, E. A., F. T. Sulzer, and J. C. Lamb, "Population Dynamics and Selection in Continuous Mixed Cultures." Journal Water Pollution Control Federation, 38, 1398-1409 (1966).

100. Lineweaver, H., and D. Burk, "The Determination of Enzyme Dissociation Constants." Journal American Chemical Society, 56, 658-666 (1934).

101. Monod, J., "The Growth of Bacterial Culture." Annual Review of Microbiology, 3, 371-394 (1949).

102. Standard Methods for the Examination of Water and Wastewater. 11th Ed. American Public Health Association, New York (1961).

103. Standard Methods for the Examination of Water and Wastewater. 12th Ed. American Public Health Association, New York (1966).

104. "Glucostat for the Enzymatic Determination of Glucose." Worthington Biochemical Corporation, Freehold, New Jersey (1963).

105. Komolrit, K., "Biochemical Response of Activated Sludge Process to Organic Shock Loads," Ph.D. Thesis, Oklahoma State University (1965).

106. Roe, J. B., "A Colorimetric Method for the Determination for the Determination of Fructose in Blood and Urine." Journal of Biological Chemistry, 107, 15-22 (1934).

107. Bhatla, M. N., "Studies on the Kinetics and Mechanism of Phasic Oxygen Uptake with Special Regard to the BOD Test." Ph.D. Thesis, Oklahoma State University (1965).

108. Ramanathan, M., "Kinetics of Completely-mixed Activated Sludge." Ph.D. Thesis, Oklahoma State University (1966).

109. Gaudy, A. F. Jr., K. Komolrit, and M. N. Bhatla, "Sequential Substrate Removal in Heterogeneous Populations." Journal Water Pollution Control Federation, 35, 903-922 (1963).

110. Gaudy, A. F. Jr., E. T. Gaudy, and K. Komolrit, "Multicomponent Substrate Utilization by Natural Populations and a Pure Culture of Escherichia coli." Applied Microbiology, 11, 157-162 (1963).

111. Wilson, I. S., and M. E. Harrison, "The Biochemical Treatment of Chemical Wastes." Journal Industrial Sewage Purification, 3, 261-275 (1960).

112. Halvorson, H. O., "Rapid and Simulataneous Sporulation." Journal Applied Bacteriology, 20, 305-314 (1957).

113. Strecker, H. J., "Formate Fixation in Pyruvate by Escherichia coli." Journal Biological Chemistry, 189, 815-830 (1951).

114. Maxon, M. D., and W. J. Johnson, "Aeration Studies on the Propagation of Baker's Yeast." Industrial Engineering Chemistry, 45, 2554-2565 (1953).

115. Horwitz, S. B., and N. O. Kaplan, "Hexitol Dehydrogenase of Bacillus subtilis." Journal Biological Chemistry, 239, 830-838 (1964).

116. P. Krishnan, "Biochemical Response of Continuous Flow Activated Sludge Processes to Quantitative Shock Loadings." Ph.D. Thesis, Oklahoma State University (1966).

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