STUDIES ON THE KINETICS AND MECHANISM OF PHASIC OXYGEN UPTAKE WITH SPECIAL REGARD TO THE BOD TEST

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CHAPTER I

INTRODUCTION

The assessment of pollution is a very vital part of sanitary engineering practice. One of the most widely accepted and used parameters for the measurement of organic pollution is a non-specific test based on the biological oxygen demand characteristics of the waste water. This test is known as the biochemical oxygen demand (BOD) test. The BOD of a waste represents the molecular oxygen consumed by a microbial population during the degradation or stabilization of organic material under standard conditions. The 5-day BOD test used as a standard procedure for comparing the strength of waste waters consists of incubating the waste sample in appropriate amount with properly seeded, air saturated and mineral fortified dilution water in airtight bottles at 20° C. The oxygen depletion in excess of the seeded blanks at the end of the 5-day period is denoted as the 5-day BOD of the waste.

The BOD reaction although simple in concept, often yields enigmatic results and has been a subject of continuing research since its inception. In the words of Hoover, Jasewicz and Porges, "No one appears to consider it

adequately understood or well adapted to his work" (1). In spite of wide criticism, the BOD test, for organic pollution measurement, is an indispensable one. It is the only microbiological assay technique available which nearly simulates conditions prevailing in the biological degradation processes during self-purification of streams, and thus should be potentially capable of yielding results of direct interpretive value. In addition, the entire framework of pollution abatement programs, i.e., pollution surveys, treatment facilities design, operation and control of treatment facilities, regulatory pollution work and the oxygen sag curve analysis, is based upon the BOD test. It would suffice to emphasize here that much research effort is needed to comprehend the nature of the biochemical oxidation occurring in low energy systems, i.e., the BOD bottle.

The earliest kinetic formulations, based on experimentation with municipal wastes, assumed the rate of deoxygenation during the course of carbonaceous BOD (oxidation of carbonaceous material excluding nitrification pertaining to oxidation of ammonia) exertion to follow first order reaction kinetics. The validity of the monomolecular principle for BOD progression for industrial wastes and pure organic compounds has been objected to by numerous investigators including the man who proposed it. It is interesting to note here that the above described kinetic concept for the microbial oxidative activity occuring in the BOD bottle

does not resemble in any way the commonly reported metabolic kinetics for bacterial cultures. Since the formulation of the first order kinetic model was based on plots of the observed data, perhaps the complexity of the seeding population, i.e., various bacterial and predator species, may be an important factor in the observance of the first order BOD plots.

As a result of studies during the last two decades, most investigators in the sanitary engineering field now agree that bio-oxidation proceeds in two distinct phases: the first, a rapid phase, is due primarily to nutrient assimilation by the cells, and the second phase represents the slow endogenous oxidation of cell substance. Endogenous oxygen uptake is assumed to occur during the entire incubation period. Recently a close examination of the curve of BOD exertion has revealed the existence of two phase oxygen uptake, with the two phases separated by a stationary pause commonly known as a plateau. It should be pointed here that, among other reasons, the major factor for the omission of the observance of this type of kinetics in the past had been due to the use of longer sampling intervals (of the order of one day), and the tendency to fit the data obtained to the first order kinetics. The generality of the existence and occurrence of this type of BOD kinetics under a variety of conditions has been definitely verified by various investigators in the bioengineering laboratories of

Oklahoma State University, Stillwater, Oklahoma (2)(3)(4)(5)(6)(7). This type of oxygen uptake expression has been observed also in high energy systems (Warburg studies).

The purpose of undertaking the present investigations was to delineate the cause(s) for the observance of diphasic oxygen uptake, separated by a plateau, and more specifically to delineate the mechanism(s) for the existence of the plateau in the oxygen uptake curve during the BOD test. It was the intent of these studies to arrive at a unified concept explaining the occurrence of this type of kinetics. The need for this research is, quite obviously, to add to the knowledge concerning the BOD reaction and to provide a sound theoretical understanding of the various phases of BOD exertion. A better evaluation of the BOD reaction will help in developing a new kinetic model or an improved BOD test and a better understanding of biological treatment. The work reported here was carried out in continuation of the studies initiated by the author some two and one-half years ago. The major channel of investigation was based on the testing of the four theories for the mechanism of occurrence of the plateau proposed earlier by Gaudy and Bhatla (5). The investigations have been conducted in both high (Warburg) and low energy (the BOD bottle) systems. A mixed population system as well as a pure culture system was studied in order to define the mechanism for the occurrence of phasic oxygen uptake. In addition, the effect of a multicomponent carbon source medium on the BOD curve was studied.

CHAPTER II

LITERATURE REVIEW

The biochemical oxygen demand test has, through a long history of development, reached its present status as a major analytical tool in the pollution control field. An extensive literature review on the development of this test has recently been compiled by O'Brien and Clark (8). Thus only a brief historical review is presented here.

Frankland is credited for first using the biochemical oxygen demand test in 1870, although the (chemical) oxygen absorption test utilizing a potassium permanganate technique for the determination of oxidizable matter was adopted much earlier by Forchamer in 1849 (9). The method used by Frankland for running the biochemical oxygen demand test resembled the present day procedure except that he determined oxygen content in the samples by gas mixture analyses obtained by boiling samples in a vacuum. These tests were run over a period of seven days.

The biochemical nature of the test was first established in 1884 by Dupre, who suggested that microorganisms which he called "microphytes" consume the dissolved oxygen for their metabolic processes (9). Dupre was also the

first of many to compare the biochemical oxygen demand and the oxygen consumed tests.

In 1888 Winkler proposed the chemical method for analysis of dissolved oxygen which now has become a part of our present day BOD test (9). The Rideal-Stewart modification of the Winkler method for use in the presence of organic matter and nitrites was published in 1901 (10). Alsterberg's azide modification of the Winkler method was not adopted until 1938 (11).

Initial efforts in understanding the biochemical nature of sewage oxidation were presented in a report of the Royal Commission on Sewage Disposal released in 1913 (9). Adney, who had already delineated the carbonaceous oxidation stage and nitrification stage of oxidation in polluted water, was one of the major contributors to this report (12). It was in 1913 that the Royal Commission recommended as standard the use of the excess oxygen method suggested by Adney (13). This method consisted essentially of the dilution bottle technique used today. Although many studies were made during this time on the occurrence of the nitrification stage, a comprehensive treatment of nitrification phenomena with relation to carbonaceous oxidation was presented by Heukelekian in 1942 (14).

Phelps in 1909 formulated a mathematical model describing the deoxygenation curve generated during the exertion of BOD (15). This effort was based on his expe-

riences with the forerunners of the dilution method, namely, the putrescibility and the relative stability tests. He made the assumption that "reaction of biochemical oxidation proceeded according to the well known law of physical chemistry governing the progress of a monomolecular reaction" which implied that "the rate of the biochemical oxidation of organic matter is proportional to the remaining concentration of unoxidized substance measured in terms of oxidizability." Theriault found an excellent agreement between the Phelps monomolecular formulation and the experimental rates of deoxygenation for sewage and industrial wastes (9). He proposed a rate constant of k = 0.1 for biochemical oxidation based on statistical analyses of his observations on Ohio river water. Theriault also developed the first order concept into equations in use today. Since then various modifications of the Phelps formula have been developed for calculation of the rate constant k and the ultimate oxygen demand L (16)(17). Orford and Ingram presented an entirely different approach for the analysis of the BOD curve (18). They adopted a logarithmic equation to fit the BOD data. The biological oxidation curve was defined by two new parameters (constants) rather than k and L used in the conventional monomolecular equation.

In the years following Theriault's work research was directed toward perfecting the dilution technique to obtain uniform and reproducible results. A major contribution of

this research effort was the development of fortified dilution water proposed by Lea and Nichols in 1936 (19). Finally, in 1946, the dilution technique for biochemical oxygen demand determination was incorporated into the 9th edition of the Standard Methods for the Examination of Water and Wastewater. A very valuable addition was made to the BOD analytical method in the 10th edition of Standard Methods based on the work of Sawyer, Callejas, Moore and Tom (20). These workers developed a set of primary standards for BOD work to indicate the presence of toxicity, deficiency of nitrogen or phosphorous in the dilution water, and the adequate viability of seeding material.

It is interesting to note that the 5-day BOD test came into use in England because of the fact that all the rivers in England have a flow time less than five days before reaching the sea. Thus the 5-day period was adopted for reasons of safe estimation of the maximum possible oxygen depletion in streams. However, much of this significance was lost after its adoption in the United States, since many rivers in this nation have more than a 5-day flow time to reach the ocean. In spite of this loss of significance, the 5-day BOD test has been retained although many attempts have been made to shorten the test time.

Apart from the research on the development of standard techniques to obtain reproducible results, efforts have been expended for many years in augmenting the understanding of

the nature and the biological significance of the BOD reaction. The investigations had two major aims: 1) a possible shortening of the BOD test; 2) comprehension of the BOD reaction leading to a better definition of its kinetic concepts. This was necessary because Phelps' mathematical treatment for the carbonaceous BOD curve, although handy and appealing in a gross engineering sense, had often been criticized and shown to be an inadequate theoretical basis for a biochemical reaction. Phelps fully realized the shortcoming of the monomolecular principle for the BOD reaction when he stated in his book "there are no obvious reasons why a biochemical reaction of this sort should proceed in accordance with the monomolecular formula. The implication is that the metabolic activities of the bacteria proportion themselves exactly to the concentration of available organic matter remaining at any time" (21).

A rigorous examination of the literature reveals that a multiphase carbonaceous BOD expression was observed many times by researchers working with the BOD test. Some investigators tried to seek explanation for this, and in doing so a large volume of criticism against the first order reaction concept was built up.

In 1926 Greenfelder and Elder noted a two-stage deoxygenation curve when incubating 20 per cent Illinois river water at 20°C. (22). The authors distinguished the second stage from nitrification and suggested that the

second deoxygenation stage was caused by an increase of putrescible matter resulting from the death of plankton which remained alive in the bottles for some time. In the same year Greenfelder, Elder and McMurray presented a comparison of the deoxygenation curve and the bacterial count using diluted sewage (23). A long lag period of several days was observed in the bacterial growth as well as oxygen uptake. While a two-stage deoxygenation was discernible from the sampling points, a rapid dieoff in bacterial count was noted after reaching a sharp peak. The peak in bacterial count corresponded to the break in oxygen uptake.

Muller in 1911 reported a correlation between bacterial growth and oxygen uptake (9). He observed that maximum oxygen depletion per hour coincided with the maximum bacterial count. Theriault in discussing Muller's findings stated that "When the bacterial count remains fairly stationary only 1/5 to 1/25 as much oxygen is used up per million bacteria as when the bacteria are in an active state of multiplication" (9).

In 1918 Purdy and Butterfield concluded from their studies in the BOD bottle using a synthetic waste inoculated with pure cultures isolated from sewage that oxygen uptake took place only while the bacteria were multiplying, and that oxygen absorption ceased after a limiting number of bacteria had been reached (24). Upon further incubations (30-40 days) they did not notice any significant oxygen

uptake even though a very high bacterial population was present. An oxygen consumption in the order of 0.01 mg/l of O_2/day/million cells was computed during the resting period.

In studies using 5 mg/l each of dextrose and peptone and a pure culture inoculum of Bacterium aerogenes, Butterfield reported bacterial cell multiplication up to 2 to 3 days accompanied by a rapid oxygen consumption (25). However, no dieoff in bacterial count was observed once a steady bacterial population was attained. Oxygen uptake also showed no significant increase after reaching the limiting bacterial count. Lea and Nichols followed the course of carbonaceous BOD exertion, viable cell count and substrate removal (19). Daily sampling over a five-day period showed that the bacterial count and substrate removal curves were generally of the same shape as the BOD curve. The viable count increased steadily at a decreasing rate, reaching a maximum population at the close of the experiment (5 days). Ninety per cent of the glucose substrate was reported to be utilized in five days. Gotaas studied the effect of various dilutions of sea water on the biochemical oxidation of sewage (26). His results showed, in almost all experiments, a very conspicuous second state of oxygen uptake. Increasing concentrations of sea water were observed to retard the secondary oxygen uptake. The author ascribed the second stage of oxygen uptake to nitrification, although no experimental evidence was presented.

Hoover, Jasewicz and Porges working with milk wastes found BOD progression to take place in two distinct phases (1). The first was a rapid assimilation phase of cell multiplication; this was followed by a slower endogenous phase involving utilization of intracellular products synthesized during the assimilation phase. According to them the BOD curve is described by two successive first order decreasing rates. They noticed that the first phase was completed in a maximum of 24 hours, and the second phase lasted throughout the incubation period. The value of the rate constant ($k = 0.1$) commonly used was suggested to be that of the endogenous phase reaction.

BOD plots for institutional sewage at 10^{o}, 20^{o} and 30^{o}C. presented by Orford, clearly showed two phases of carbonaceous BOD exertion; i.e., two phases separated by a pause (27). Because the samples were taken at one day intervals, the pause between the two phases was not clearly emphasized.

Buswell, Mueller and Van Meter have also recognized a two-stage oxygen uptake during carbonaceous BOD exertion (28). One was associated with cell multiplication, and the second was related to a resting or dying bacterial population. They did not suggest any simple relationship between substrate and rate of oxygen utilization in either stage.

Zehnpfennig and Nichols, trying to develop a two-day BOD test, visualized the BOD reaction to occur in two

phases (29). The first phase, proposed to be controlled by substrate concentration, followed first order kinetics, and the second phase was interpreted as being controlled by the reproduction rate of bacteria due to predator action in the later part of the BOD test. This phase followed a linear relationship.

BOD curves reported by Tidwell and Sorrels exhibited a secondary rise in oxygen uptake (30). Their work was accomplished using a pure culture of Pseudomonas aeruginosa. The maximum bacterial count corresponded to the end of the first stage of oxygen uptake, and did not decrease in an appreciable amount during their short term experiment.

During development of a short term BOD test in 1958, Busch reported a diphasic carbonaceous BOD curve with two phases of oxygen uptake separated by a plateau (31). He attributed the first phase of oxygen uptake to the conversion of substrate into cell substance which included new cell synthesis and storage products, and associated the second phase of oxygen absorption with the oxidative endogenous metabolism of bacteria. Oxygen utilization during the second phase in excess of the respiratory activity of bacteria was attributed to the action of predators; e.g., protozoa feeding on bacteria. The theory postulated by Busch is similar to that of Hoover et al. except for the existence of the plateau between the two phases and the involvement of predators (1). According to Busch "as the

ratio of predators to substrate-consuming population increases, the separation of two stages begins to blur." He noted a reduction of second stage oxygen uptake after treatment of the seed to reduce the protozoan population. However, the author failed to state clearly any cause for the occurrence of the plateau.

Myrick and Busch were successful in artificially increasing the plateau value, i.e., first stage of oxygen uptake, by selectively blocking the assimilation process with small quantities of sodium azide (32). They also observed multiphase (including the plateau) BOD kinetics in high substrate concentrations in the Warburg apparatus using glucose, glutamic acid, and a mixture of both.

Tsivoglou in analyzing BOD data on Kanawha River water found that the two component first order reactions best fitted the observed data (33). While applying this concept to other data, he noticed a break between the two phases of oxygen uptake on the second or third day, producing a BOD curve similar to that observed by Busch.

Lee and Oswald have studied the course of BOD exertion and population dynamics using sterile sewage (34). Their oxygen uptake rate data calculated for two-hour and six-hour intervals show a very rapid rise followed by a sharp decrease. The bacterial count attained a peak at 24 to 36 hours, somewhat later than the maximum oxygen uptake rate. Based on the 6-hour oxygen uptake rate, these workers

related the oxygen absorption with three phases of bacterial growth. The first phase of rapid oxygen uptake rate was attributed to cell growth involving conversion of available nutrients into cell mass. However, they believed that the new growth as well as the old cells remained in clumps or flocculent masses, and therefore the lag between the maximum rate of oxygen uptake and the maximum bacterial count was observed. The second phase was associated either with "swarming" or mechanical or chemical dispersion of clumps, resulting in a higher count than the first phase. Cell die-off, constituting the third phase, occurred due to the non-availability of nutrients for the dispersed cells. No plateau was evidenced in their accumulated oxygen uptake curves; however, the examination of their plots of oxygen uptake rate suggested a period of low activity between the rapid and slow phases of oxygen uptake.

In pure culture work Halvorson observed two stages in oxygen absorption connected by a period of low oxygen demand, employing the active culturing technique for rapid growth and simultaneous sporulation of Bacillus cereus var. terminalis (35). Cells were grown on glucose-yeast extract-mineral media. While the first stage of oxygen uptake was due to the vegetative growth of cells, the initiation of the second stage of oxygen uptake was characterized by sporogensis. The investigator proposed that this pattern of oxygen absorption was probably due to

glucose interference with adaptive enzymatic processes needed for the complete oxidation of the glucose catabolites, mainly acids in nature. Nakata and Halvorson, later investigating the same system, reported the accumulation of pyruvic and acetic acids in the medium during the vegetative growth of the cells, corresponding to the first stage of oxygen absorption observed by Halvorson (36). The second stage of oxygen absorption was accompanied by rapid utilization of these acids by sporulating cells. It was suggested that the transition phase in oxygen uptake was due to the induction of an acetate oxidizing enzyme system. Recently Hanson, Srinivasan and Halvorson have shown the absence of an enzyme system responsible for the terminal oxidation of acetate to CO_2 in the vegetative state of Bacillus cereus strain T (37). They have further found that induction of the enzymes of the TCA cycle is inhibited by addition of chloramphenicol during the early stages of sporulation.

In an attempt to test Busch's hypothesis pertaining to the contribution of protozoa to the second stage of oxygen uptake, Wilson and Harrison demonstrated a two-stage oxygen uptake curve, i.e., two stages separated by a plateau, using a pure culture inoculum in phthalic acid solution as a sole carbon source (38). This work was conducted with fairly high substrate concentration in a Warburg apparatus. These workers related the magnitude of the secondary rise in oxygen utilization to the number of

bacterial cells present in the system. They identified the second stage of oxygen uptake with endogenous respiration; however, no reasons were cited for the delayed onset of the endogenous activity. The authors concluded that pretreatment of the inoculum, practiced by Busch to reduce protozoa, also caused selection of bacterial species, and hence a reduced secondary oxygen uptake.

Abu-Niaaj observed a plateau in the BOD bottle using glucose as substrate. In only one experiment performed with nutrient broth as substrate the plateau was not observed (2). The peak in bacterial population was correlated with the plateau in the oxygen uptake curve.

McWhorter and Heukelekian presented data on oxygen uptake in high energy systems (Warburg experiments) using sewage seed (39). They observed the existence of the plateau at glucose concentrations of 100 and 500 mg/l, but not at 1000 mg/l. In determining the effect of protozoa on oxygen uptake, they did not find any difference in the magnitude of oxygen uptake for sonicated and normal seed systems until 45 per cent of the theoretical oxygen demand had been exerted. Since this was more than the plateau value for glucose (41 per cent) suggested by Busch, it was surmised that the disappearance or masking of the plateau at high concentrations of glucose could not be due solely to the changes in predator-bacteria ratio in the initial inoculum (40). However, no explanation was presented for the appearance and disappearance of the plateau.

Marion and Malaney in their studies on oxidation of aliphatic compounds by Alcaligenes faecalis (Warburg apparatus) observed a plateau in oxygen uptake in most of their experiments, both in endogenous and sample flasks (41). They suggested that it was similar to a diauxic effect caused by acclimation to two successive intracellular substrates.

In research in the bioengineering laboratories of Oklahoma State University, Gaudy, Bhatla and Abu-Niaaj, Gaudy and Bhatla, and Follett and Gaudy have established the generality of the occurrence of a plateau during BOD progression under a variety of seeding and substrate conditions (4)(5)(6). Viable bacterial counts were estimated during these studies. Gaudy, Bhatla and Abu-Niaaj, and Gaudy and Bhatla also observed a plateau with a pure culture prototrophic strain of Escherichia coli using glucose as sole carbon source in the BOD bottle. In general, in all of these studies in our laboratory the first phase of rapid oxygen uptake was accompanied by an increase in bacterial numbers, the plateau corresponded to the range of steady bacterial population, and the secondary rise in oxygen uptake was marked by a decrease in viable bacterial count.

Based on experiences with the BOD test, Gaudy and Bhatla have proposed four related theories for the causation of the plateau (5). These theories have one central likeness: the plateau is caused by a shift from metabolism of the original exogenous carbon source to biologically

synthesized intra- or extra-cellular substrates. Bhatla and Gaudy in recent work on the investigation of reason(s) for the existence of the plateau have eliminated two theories of the four previously postulated as being of possible general applicability to the heterogeneous population systems (7). This work was accomplished in high substrate concentrations (Warburg) using synthetic waste.

A more detailed account of works contributed by the writer is presented in the "Theoretical Concepts", Chapter III, of this dissertation.

Influence of Protozoa on Biological Oxidation

As early as 1918 Purdy and Butterfield, investigating the effect of plankton on bacterial death rates, noticed very little visible purification in the bacterial system in the absence of plankton (24). When plankton were also present, destruction of large numbers of bacteria was recorded and the process of purification was apparently completed. The term "purification" was considered to be synonymous with clarification attained in the samples, since no chemical tests for estimating the purification of samples were made.

Waksman discussed the interaction of protozoa and bacteria with regard to soil fertility (42). He stated that protozoa enhance the fertility of soil by destroying excess numbers of bacteria thereby allowing further development of

bacteria, the prime reasons for biological transformations in soil. Investigations by Russell and Hutchinson on soil sickness and partial sterilization of soil suggested protozoa as probably inimical to the useful aerobic bacteria and hence to soil fertility (43).

Butterfield, Purdy and Theriault noticed no difference in the shape of the BOD curve using pure cultures of bacteria and mixed cultures of bacteria, except for somewhat greater oxidation in the latter case (44). In both cases a rapid phase of oxygen uptake, corresponding to the bacterial growth period, was followed by a very slow phase of oxygen uptake. However, on addition of the protozoan Colipidium to a pure culture of Bacterium aerogenes, active oxygen uptake continued much beyond the peak in the bacterial population, resulting in almost twice as much oxygen depletion as in the bacterial control. The bacterial population did not quite reach the count attained in the bacterial control, but showed a rapid decline in bacterial numbers after one day followed by an increase in protozoa count which reached a peak after four days. The bacteria count in the control did not decline significantly after reaching a ceiling value. Based on their investigations, Butterfield et al. advanced a theory stating that "The chief function of certain plankton in the biochemical oxidation process is to keep the bacterial population reduced below a saturation point and thus to provide conditions

suitable for continuous bacterial multiplication, this in turn resulting in a more complete oxidation."

Phelps endorsed the hypothesis of Butterfield, Purdy and Theriault (21). He regarded bacteria as the main agents in sewage purification, and maintained that the rate of purification is determined by the growth rate of bacteria rather than their numbers. Accordingly, Phelps' interpretation of the role of protozoa in purification processes is that they serve to keep the bacterial populations at levels which can accomplish maximum work of oxidation.

Other investigators have pointed out the distinction between the chemical purification, i.e., BOD removal, and the visible purification, i.e., clarification, and have noticed a higher clarification in the presence of protozoa (45)(46). According to these workers the protozoa did not effect chemical purification. Protozoa were considered of secondary importance in the total process of purification by activated sludge.

Cramer concluded from his results that protozoa were necessary for clarification of sewage by an activated sludge (47).

Liebmann noticed no difference in the rate of purification in the presence or absence of protozoa (48)(49). He argued against the view held by Butterfield et al., and Phelps. He stated that the number of bacteria in a system is determined by the available food concentration only,

and consequently suggested that there is no possibility of "overdevelopment" of bacteria by predation activities of protozoa.

Pillai and Subrahmanyan considered protozoa as major participants in aerobic sewage purification, with bacteria playing a secondary role (50). These workers demonstrated better removal of chemical oxygen demand (COD) using a pure culture of protozoa (Epistylis sp.) than was obtained using activated sludge or mixed cultures of bacteria utilizing sterile sewage as substrate. They also found that heating of activated sludge to 50°C. not only killed protozoa but also stopped clarification of sewage by an activated sludge process.

Zehnpfennig and Nichols observed nearly 30 per cent less 5-day BOD when protozoa were removed from the sewage seed by straining (29). Autoclaved sewage and pulp mill waste were used as substrates in these studies.

Javornicky and Prokesova have recently studied the influence of protozoa on the BOD process (51). These investigators observed high reduction in bacterial population during the development of protozoa in the BOD bottle, showing a simultaneous increase in oxygen uptake. In studies using "partially sterilized water" to selectively kill the predators they noticed higher bacterial count and lower oxygen uptake. They thought that their results were explained by the hypothesis of Butterfield, Purdy and

Theriault. Javornicky and Prokesova also placed a pure culture inoculum of protozoa, Tetrahymena pyriformis, into the mixed bacterial system. The protozoa apparently did not flourish and did not effect oxygen uptake. In other experiments minute flagellates up to 10 microns in size caused only a slight decrease in bacterial number, and a very small increase in oxygen consumption. They considered that both the quantity and quality of protozoa are important in the determination of the BOD. Their oxygen utilization data showed considerable fluctuation, and no rigid treatment of the BOD kinetics could be made.

CHAPTER III

THEORETICAL CONCEPTS

Since the present study constituted the concluding phase of the investigations undertaken to resolve the controversy concerning phasic phenomena in oxygen uptake pertaining to the BOD test, it was considered appropriate to include this section in order to present in reasonable detail the concepts already developed based on the past investigations of the author, and to review the status of the investigations prior to the work contained in the dissertation. Much of the earlier work reviewed in this section was conducted by the author, but is not formally included in this dissertation in order to conserve space and because these investigations have already been published (3)(4)(5)(6)(7).

In general, two experimental approaches have been adopted in the investigations of this problem. First it was necessary to establish the range of conditions under which the plateau occurred between the two phases of oxygen uptake and the generality of its occurrence. In attainment of this aim approximately fifty long-term BOD experiments were conducted in which the time course of

BOD exertion and changes in the viable bacterial populations were studied. Investigations covered four different types of seed materials, namely, settled sewage, washed acclimated cells, supernatant from a laboratory scale batch activated sludge unit, and a washed glucose acclimated seed consisting of a pure culture of Escherichia coli. Experiments were conducted using a variety of pure compounds and mixtures of pure compounds as carbon sources. Studies were also made on complex substrates such as sterile sewage, nutrient broth, digester liquor from a neutral sulfite semi-chemical pulp operation, and Kraft mill (digester) black liquor. In addition, the effects of substrate concentration and initial seed concentrations were also studied. In the great majority of the systems tested a plateau was discerned between two distinct oxygen uptake phases. In general, at low initial seed populations which allowed an increase in bacterial numbers, the first rapid period of oxygen uptake corresponded to rapid cell multiplication. The plateau in oxygen uptake corresponded to the range of the maximum bacterial population, and the duration of the plateau was observed to be somewhat proportional to the length of the stationary phase in maximum viable population. The second phase of oxygen uptake corresponded to a decline in viable bacterial population. Typical results are shown in Fig. 1. The decrease in viable bacterial count during the second stage of oxygen uptake was not nearly as rapid using pure

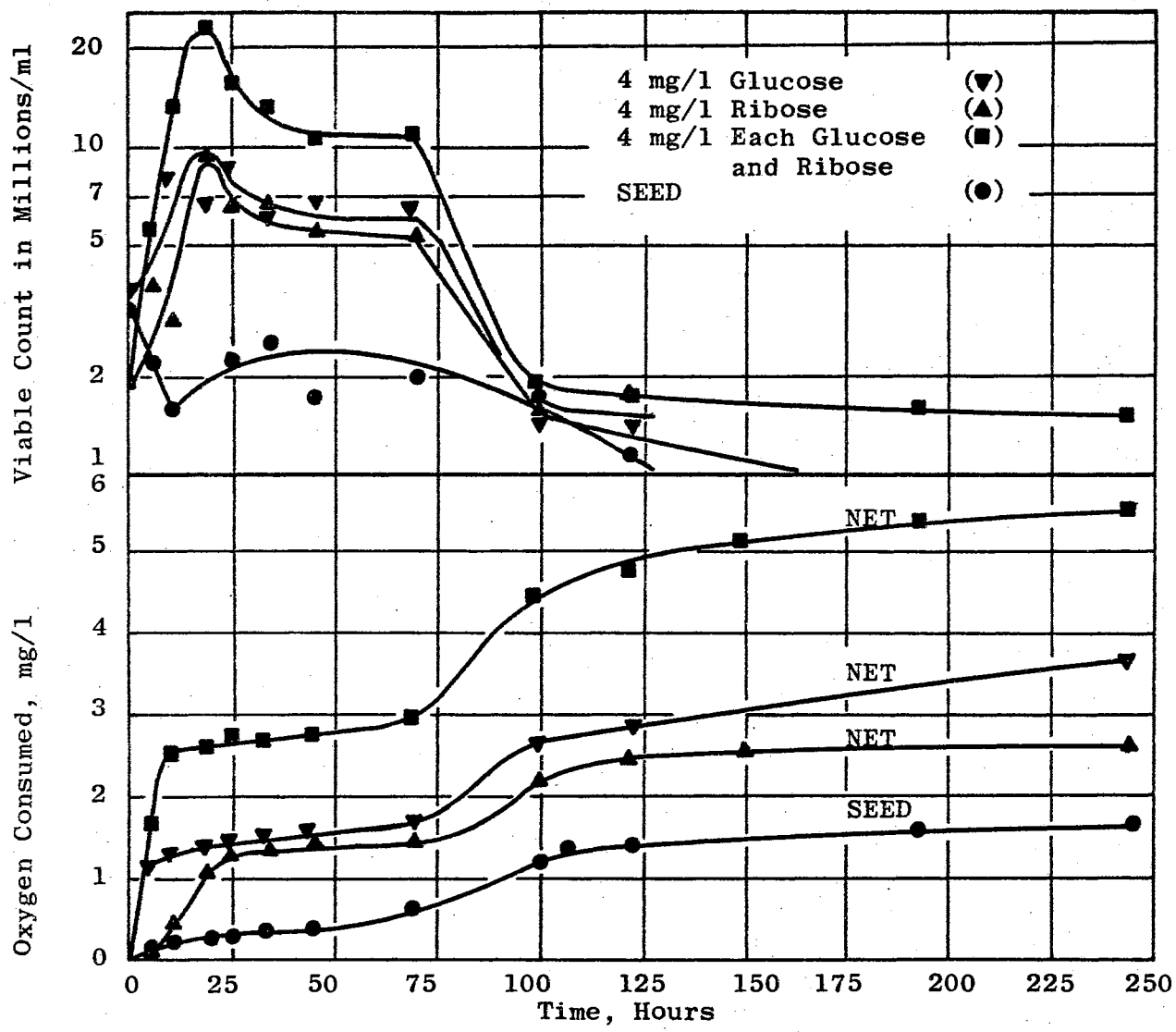


Fig. 1 - BOD AND VIABLE BACTERIA COUNT, SEED TYPE C ACCLIMATED TO GLUCOSE.

culture seed as with mixed population seeds. Phasic oxygen utilization was also recorded in some seed blanks purposely held devoid of any substrate; this was especially true when oxygen consumption by the seed blank was high. From studies such as those described above it was concluded that diphasic BOD expression separated by a pause or plateau was of general occurrence under a wide variety of conditions.

Based upon the results obtained in dilute systems (the BOD bottle), four mechanistic theories of causation for the plateau during BOD progression were formulated.

The second approach in these investigations was focussed on the delineation of the causes for the existence of the plateau. This was systematically attempted by testing the applicability of the four proposed theories. Before any further research effort in this area is reviewed it is desirable to describe the four theories formulated by Gaudy and Bhatla, because all the later work was conducted strictly within the scope of these theories (5).

Theory 1

In heterogeneous populations a certain set of bacterial species predominate and attain a maximum population determined by the environmental conditions and amount of available substrate. Upon exhaustion of the substrate the predominating cells begin to die off and may release cellular components which can become substrate for other less prevalent species in the system. This abundance of sub-

strate created by the death of the predominating species in relation to the small number of cells which can use these cellular components may result in an autocatalytic type growth curve of these latter cells, resulting in a second oxygen uptake curve and hence creating a plateau in oxygen uptake expression at the end of the first growth curve and start of the second growth curve. Alternatively, the cells themselves may become food for protozoa. In this case the second stage of oxygen uptake may be an expression of the respiratory requirements of protozoan metabolism. The plateau, as in the case of a secondary bacterial predominance, represents the end of one growth curve and the beginning of another.

Theory 2

This theory differs from the first theory in that no change in predominance takes place after the maximum cell population is attained. Instead, a portion of the cells die and the cellular components released in the medium serve to sustain the viability of the remaining population. However, since the new substrates are of very different configuration than the original substrate, an acclimation period is required in order to synthesize new enzyme(s) or build up the level of existing enzyme(s) to metabolize the new, now exogenous substrates. The exogenous food produced may not be adequate to support a detectible net growth, thus accounting for the observed slow dieoff of cells.

Theory 3

The cells lose their replicating ability after reaching maximum growth supported by the system; however, they continue to respire. An acclimation time may be required before cellular components previously synthesized during active metabolism of the original substrate are utilized as sources of energy. Another possibility within this theory is that an acclimation period is needed before the cells can use certain storage products for energy. The existence and length of the plateau in such systems will depend upon the nature of the storage products and the induction time, if required, for manufacturing the necessary catabolic enzymes. Reactions of this nature may provide an explanation for the delayed onset of endogenous respiration suggested as a possible cause for the plateau by Wilson and Harrison (38).

Theory 4

Metabolism of some compounds may yield exogenous intermediate compounds instead of direct assimilation as new cells and intracellular storage products. In such cases a lag period, reflected as a plateau in the oxygen uptake curve, may be required for the induction of enzyme(s) to initiate metabolism of the newly formed exogenous compounds. Even though in some cases the cells may already possess the enzymes needed to metabolize the intermediate compounds formed, new transport enzyme(s),

permeases, may have to be acquired to transport these compounds to active sites of existing enzymes inside the cell. Cells may start to die off due to lack of ready and adequate availability of energy source. This lack of ready energy source could lead to a pause in oxygen uptake expression.

Gaudy and Bhatla believed that all four theories could contribute to producing the plateau in some systems; however, Theory 1 was obviously eliminated as a general application since pure cultures exhibited a plateau.

These four theories provided the foundation upon which was laid the framework for future investigations seeking greater insight into the causes for existence of the plateau. Work was continued along this line by Bhatla and Gaudy in high energy (Warburg) systems using mixed populations and synthetic waste containing glucose as sole carbon source (7). High concentration systems were employed because it was advantageous to work at as high a concentration of substrate as possible to make available sufficient materials (substrate and cells) for the study of certain biochemical parameters. This obviated the need to devise highly specialized techniques and elaborate experimental design which might have been necessary to study the systems in the BOD bottle due to the microconcentrations of cells and substrate involved. Two phases of oxygen uptake were shown to exist regardless of substrate concentrations from 100 - 1000 mg/l or initial solids concentrations from 8 to

500 mg/l; however, the lag between the two phases was noticeably diminished by using higher substrate or initial solids concentrations. It was felt that the merging of the two phases of oxygen uptake was probably associated with a higher endogenous respiration in the high substrate or the high initial solids systems; both systems possessed higher endogenous oxygen requirements by virtue of the larger amount of cells participating in the process. Substrate concentration did not materially affect the time of occurrence of the second phase, but an increase in solids concentration tended to hasten the occurrence of secondary oxygen uptake. Based on these preliminary considerations (i.e., effect of substrate and initial solids concentration on the plateau), 500 mg/l of substrate and a very small initial inoculum of cells was chosen as a model system for subsequent investigations. Possible relationships between different phases of oxygen uptake and parameters such as substrate removal (measured by the COD and anthrone tests), intracellular protein content and solids production were examined. Efforts were also made to determine whether substrates not responsive to the COD test (i.e., certain volatile acids) were secreted into the medium. From the results of this experimentation it was concluded that lysis of a portion of the bacterial population with subsequent reuse of cellular components after an acclimation period (Theory 2) could not be considered a general cause for the

plateau in the heterogeneous populations studied. Also, it was suggested that Theory 4, involving the excretion of intermediates or end products during the metabolism of the original compound could not be cited as a general reason for the occurrence of the plateau in heterogeneous population systems. These findings were supported by the fact that systems devoid of exogenous substrates (i.e., endogenous blanks) also exhibited phasic oxygen uptake.

The results of the above studies strongly pointed toward the role of Theory 1 concerning population predominance and Theory 3 involving reactions within the biomass as being responsible for the causation of the plateau. It was, however, emphasized that the above logical conclusions for the cause of the plateau were potentially applicable to heterogeneous population systems only. Indeed, the theories eliminated (2 and 4) could still be invoked to explain the occurrence of the plateau in some systems (especially pure cultures).

With this as a background, the present investigations were carried out in continuation of the work of Bhatla and Gaudy to further delineate the cause for the occurrence of the plateau in mixed populations. Major research emphasis was placed on testing possibilities within the scope of Theories 1 and 3. A pure culture system was also studied to examine the cause(s) for the existence of the plateau in oxygen uptake. In addition a two-carbon source,

glucose-sorbitol, system was studied in the BOD bottle to clarify the role of sequential substrate removal in oxygen uptake in dilute systems.

Examination of Theoretical Concepts

The present research was conducted in three major phases. Since each phase was studied somewhat independently, each will be reported separately. The three phases are as follows:

PHASE I Investigations into causation of the plateau in mixed population systems.

PHASE II Investigations into the causation of the plateau in pure culture systems.

PHASE III Phasic oxygen uptake in multicomponent substrate systems.

PHASE I

Investigations in this phase of the research were limited to examinations of Theories 1 and 3.

A. Examination of Theory 1

According to Theory 1, a plateau in oxygen uptake expression may be caused by a secondary predominating species subsisting on the product of the first. The secondary predominance may be bacterial in nature or may consist of predator organisms, e.g., protozoa. One way of studying the secondary bacterial predominance would

have been to use known mixtures of organisms rather than natural populations. While this would have made an interesting study, it is the naturally selected heterogeneous population which has more direct applicability in a study pertaining to water pollution control aspects. It was therefore deemed of greater immediate value to use natural populations and to determine the role of protozoa in creating the plateau in oxygen uptake curve. A variety of experiments were designed. Methods for selectively removing the protozoa and counting protozoa were perfected. The effect on oxygen uptake of the protozoa-bacteria ratio was studied in both dilute and concentrated systems. In addition, effects of addition of protozoa to bacterial systems were examined with respect to oxygen uptake, substrate removal, and cellular protein content.

B. Examination of Theory 3

Theory 3 recognizes a requirement for an acclimation period before the cells can endogenously metabolize certain cellular components synthesized in replicating processes (e.g., excess protein or nucleic acid). This acclimation time is devoted to synthesis of new enzyme(s) needed for metabolism of the cellular compounds, and is expressed as a pause in the oxygen uptake curve. Accordingly, after reaching maximum population, protein synthesis is a prerequisite for the exertion of the second stage of oxygen uptake.

To examine the role of Theory 3 in generating a plateau an inhibitor of protein synthesis, chloramphenicol, was employed. The inhibitor was added in appropriate concentrations to the medium near the end of the first stage of oxygen uptake, thus stopping the manufacture of protein in the system. By the same token any bacterial growth after treatment with the inhibitor was prevented. This also served to check the secondary bacterial predominance as the cause for the plateau.

Many antibiotics are available for inhibition of protein synthesis but chloramphenicol, also known as chloromycetin, has been most widely used with bacteria, and its mode of action is well understood. It was ideally suited for this study because chloramphenicol has no effect on fermentation or respiration of glucose even at very high concentrations (52)(53). It is a bacteriostatic agent and has been shown to be effective in inhibiting a large variety of bacteria, from practically all families, at concentrations between 1 and 10 µg/ml (54)(55). Most protozoa are very resistant to chloramphenicol except Tetrahymena pyriformis, which is inhibited at a concentration of 40 µg/ml (54)(56). The inhibitory action is obtained through the blocking of protein synthesis. Its mode of action has been suggested to involve interference with processes of transfer of amino acid from soluble RNA to protein during protein synthesis (57).

Preliminary studies for checking the efficacy of chloramphenicol with heterogeneous populations were made before its use. Working concentration limits were also established.

PHASE II

In a very important sense it was desirable to investigate the mechanism(s) for the causation of the plateau observed in pure culture systems. One of the purposes of including this work was to try to develop sound generalized concepts for the existence of the plateau in all systems (both heterogeneous and pure culture) through logical elimination or consolidation of the proposed theories. It has been reported by various investigators that certain pure cultures also exhibit biphasic oxygen uptake separated by a plateau. For example, such studies were reported by Gaudy, Bhatla and Abu-Niaaj, and Gaudy and Bhatla, who observed a plateau in the BOD bottle using a prototrophic strain of Escherichia coli (4)(5). These results were obtained with glucose as the sole carbon source. A second study was documented by Wilson and Harrison, who observed a plateau with a heterotrophic organism (isolated from activated sludge) using phthalic acid as the carbon source (38). These studies were conducted in the Warburg apparatus at fairly high substrate concentrations. The bacterium was not identified in detail by the authors, but they felt that it was a species of Pseudomonas (58).

Neither of these pure cultures was available for study in the present investigations. No reports were available regarding observance of a plateau for pure cultures in the purely microbiological literature. The first approach, then, was an attempt to obtain a pure culture system which would exhibit a plateau in oxygen uptake. The choice of the bacterium and system conditions were limited to an organotroph capable of growing under aerobic conditions on a minimal medium using a sole carbon source. The obvious approach in accomplishing this was to obtain identified pure cultures available from the microbiology department at Oklahoma State University which could be cultured on minimal media. Glucose was elected as the carbon source because it is metabolized by a wide variety of heterotrophs; thus the choice of the organisms would not be very limited. In addition, bacteria were isolated from two laboratory activated sludge units fed on glucose and phthalic acid respectively. The units were restarted many times, using sewage seed.

After having obtained a pure culture exhibiting typical bimodal oxygen uptake, investigations were devoted to establishing the range of conditions for the occurrence of the plateau. The conditions included the effect of different substrate concentrations, initial solids concentrations, and various substrates. Long term aeration studies on the Warburg apparatus were made in which parameters such as substrate removal, intracellular protein and solids production were examined. Inhibitor studies were also made.

PHASE III

Gaudy, Komolrit and Bhatla reported a diphasic oxygen uptake in high energy systems using a multicomponent glucose-sorbitol carbon source (59). The phasic nature of the oxygen uptake curve was attributed to sequential substrate utilization. A diphasic oxygen utilization was also observed by Gaudy and Bhatla in the BOD bottle, using a glucose-sorbitol multicomponent carbon source and glucose-acclimated seed (5). On the basis of the results obtained in low energy systems and the time of occurrence of the break as well as the oxygen consumption by glucose and sorbitol controls, these workers pointed out that the pause in the oxygen uptake curve could be caused due to sequential substrate removal in the BOD bottle, although study of such phenomena has never been attempted in the BOD bottle. Indeed, sequential or phasic substrate removal cannot be cited as a general cause for the occurrence of the plateau, since the plateau occurs with a single substrate as well. However, it would be of great significance if the reported plateau in the BOD bottle using multicomponent substrate occurred due to the mechanism of diphasic substrate utilization. This line of investigation is relevant to the present study in that the sequential removal of substrate could be a valid cause for the existence of a plateau outside the scope of the four theories previously outlined. This type of experimentation was of added interest in the

comparison of dilute and concentrated systems. Substrate elimination was studied in the BOD bottle using a glucose and sorbitol system. Both glucose- and sorbitol-acclimated seeds were employed.

CHAPTER IV

MATERIALS AND METHODS

Materials and Methods are presented in three major sections: 1. Composition of synthetic waste water; 2. A description of all of the analytical techniques employed during these studies; 3. The experimental protocols. This latter portion is further subdivided into three sections encompassing the entire research effort.

Composition of Synthetic Waste Water

The inorganic constituents in the synthetic waste and their concentrations (Table I) were selected on the basis of work reported in the bacteriological field for optimum culture requirements of minimal media. The following range of COD to nitrogen and phosphorus ratios were computed using glucose as substrate:

Glucose Concentration	COD/N		COD/P
	NO_3^- - N	NH_4^+ - N	
500 mg/l	4.05	2.52	0.57
3000 mg/l	24.3	15.1	3.44

Except for the 3000 mg/l glucose concentration system using NO_3^- nitrogen the concentrations of mineral elements (nitrogen and phosphorus) used in these studies were in excess of the critical limits (BOD/N = 17 and BOD/P = 90) for optimum BOD removal generally employed in sanitary engineering practice (60). Even in the case of the 3000 mg/l substrate level the recommended ratios were not exceeded if 5-day BOD values (used in normal practice) were employed in the computations instead of the theoretical chemical oxygen demand values. The 5-day BOD in the case of glucose might be taken as 70 per cent of the COD value; this would reduce the tabulated values by 30 per cent. Furthermore, it should be pointed out here that Sawyer indicated minimum nitrogen and phosphorus requirements of BOD/N = 32 and BOD/P = 150 to accomplish stabilization of a waste (60). The very high amount of phosphorus in the system was due to the use of the phosphate buffer required to maintain the pH at 7 during the experiments. The point delineated here is that in no case was nitrogen or phosphorus a limiting metabolite; the limiting nutrient was the carbon source. Nitrogen as ammonium ion (NH_4^+ - N) was used whenever pure cultures were involved, while nitrate-nitrogen (NO_3^- - N) was employed during investigations using heterogeneous populations. The choice of nitrate over ammonium-nitrogen in mixed population studies

was made to obviate the need to consider oxygen uptake due to nitrification by autotrophs (7)(39)(61).

TABLE I

COMPOSITION OF SYNTHETIC WASTE WATER

Constituents	Quantities per Liter	
	For mixed Population Studies	For pure Culture Studies
Glucose or other Carbon Source	500 - 3000 mg*	500 - 3000 mg*
1.0 M Potassium Phosphate Buffer pH 7.0	30 ml	30 ml
$NaNO_3$	800 mg	-
$(NH_4)_2SO_4$	-	1000 mg
$MgSO_4 \cdot 7H_2O$	200 mg	200 mg
$FeCl_3 \cdot 6H_2O$	1 mg	1 mg
$MnSO_4 \cdot H_2O$	20 mg	20 mg
$CaCl_2$	15 mg	15 mg
Tap Water	100 ml	100 ml
Distilled Water	To make one liter	

*3000 mg/l concentration was used for obtaining acclimated seed.

Analytical Techniques

The following analytical techniques were used:

BOD Determination

The biochemical oxygen demand tests were run according to Standard Methods for the Examination of Water, Sewage and Industrial Wastes (62). Deionized water was used for dilution water, and was obtained by running tap water through a mixed bed ion exchange resin (Barnstead, Bantam Demineralizer). Dilution water was prepared by the addition of reagents prescribed in Standard Methods. $(NH_4)_2\ SO_4$ was used as a nitrogen source. Dissolved oxygen was determined by the Alsterberg (azide) modification of the Winkler method. Bottles were incubated in the dark at 20°C.

Oxygen Uptake Measurements

Direct oxygen absorption was measured in 125 ml Warburg vessels using 40 ml of reaction fluid and 1.5 ml of 20 per cent KOH in the center well. Shaker rate was maintained at 100 oscillations per minute, and temperature at 25°C. (77°F.) throughout the research.

Bacterial Counts

The heterotrophic bacterial population, reported as viable cells per ml, was counted by cultivation on solid nutrient agar (Difco) at 37°C. The surface plating

technique as reported by Gaudy et al. was employed, and incubation was continued until a constant number of colonies were attained (normally 48 hours of incubation) (63). The count for each sample was based on a total volume of 0.08 ml of sample appropriately diluted with sterile distilled water plated in four spots (0.02 ml per spot) on each half plate. Duplicate samples of each dilution were made, and the results reported are the average counts from the duplicates.

Protozoa Counts

Only conspicuously motile protozoa were counted. For making the count a 15 x 20 x 1/10 mm Howard Mold counting chamber was used. This counting chamber, holding 0.03 ml of sample volume, was examined for protozoa at 125 x magnification employing a Zeiss bright field microscope. This magnification exposed an area of 1.5 mm^2 for each field of view. Each counting operation consisted of counting 25 fields out of a possible number of 200. A set counting pattern was followed in order to help achieve random sampling. The results reported are the average of at least two counting sequences performed on two different volumes of each sample. Sterile distilled water was used for making dilutions when desired. Reported protozoa populations were then computed as follows:

Protozoa count in the sample = number of motile protozoa observed in 25 fields x $\frac{15 \times 20}{1.5 \times 25}$ x $\frac{100}{3}$ x dilution factor. During the microscopic examination protozoa were identified based on the morphological characteristics described by Whipple in Plates XII to XV (64).

Chemical Oxygen Demand Determination

The COD test described in Standard Methods was used to determine the oxidizable organic material during the course of substrate removal (62). Potassium dichromate (0.25N) and concentrated sulfuric acid were used. Silver sulphate was always included as a catalyst.

Biological Solids Determination

The membrane filter technique (Millipore Filter Corp., Bedford, Mass.; HA, 0.45μ) was employed for biological solids determination. Very light aluminum dishes (weighing about 0.2 gm) were used to hold the millipore filters. Filters were heated for two hours at 103°C. and equilibrated in a desiccator overnight prior to obtaining the tare weight. Before final weighing the same heating and cooling procedure was followed.

Carbohydrate Determination

The anthrone test, which is specific for carbohydrates, was employed for examining the course of glucose removal when the latter was used as a sole carbon source. The test was performed using the technique described by

Gaudy (65). A modification with respect to the quantity of anthrone later proposed by Gaudy, Komolrit and Bhatla was incorporated (59). This method was also used to determine the carbohydrate content of cells.

Periodate - Chromotrophic Acid Method for Polyalcohols

Periodate oxidation with subsequent color reaction with chromotrophic acid for determination of polyalcohols was used as a specific test for following the metabolism of sorbitol. Results were corrected for glucose interference when both glucose and sorbitol were used as the carbon source in the system. The method used was exactly the same as that reported by Gaudy, et al. (66).

Concentration of Samples

Substrate removal in the BOD bottle was studied by concentrating the filtrate (HA, 0.45 μ Millipore filter) under reduced pressure at 50°C. in a flash evaporation apparatus. Two hundred ml samples were concentrated to a volume of 5 ml for subsequent chemical analysis.

Volatile Acids Determination

Steam distillation of the acidified samples followed by titration of the distillate with 0.05N NaOH was used to determine the volatile acids in the sample. The procedure described in Standard Methods for the Examination of Water, Sewage and Industrial Wastes was used (62).

Pasteurization of Seed

Seed pasteurization as recommended by Pillai et al. and Heukelekian et al. at 50°C. for five minutes was employed for selectively killing protozoa (50)(46). The method has been re-examined and successfully used in recent investigations by Javornicky and Prokesova (51). In the present investigations the pasteurization procedure for eliminating protozoa from the sewage seed was found to be very successful; however, some difficulty was experienced in effecting complete inactivation of protozoa by pasteurization when an acclimated seed was used. It is felt that the very high cell density in the acclimated seed employed was the cause for only partial pasteurization. Three minutes exposure at 50°C. was found to be adequate to attain maximum pasteurization.

Protein Determination - Copper-Folin Method

A wide latitude is available in the choice of methods for protein determination. The Kjeldahl method for the determination of organically bound nitrogen is the most commonly employed in the sanitary engineering field. This test has usually served as a standard for comparing the efficiencies of other methods which determine the protein content based on the presence of a particular amino acid, a group of amino acids, or other common characteristics of the protein molecule such as peptide bonds. One such

colorimetric method (biuret test) based on the reaction of copper with peptide bonds of the protein molecule was adopted by Gaudy and has been successfully used for the determination of protein content of biological sludges in sanitary engineering research (65). The test is preferred to the Kjeldahl procedure on the merits of its specificity for protein and convenience in analysis of a large number of samples. The Kjeldahl method, besides being laborious and time-consuming, in its most sensitive form requires for accurate determinations 0.4 to 1.4 mg of nitrogen in the sample (67). The equivalent amount of protein required may be calculated on the assumption that protein contains 16.5 per cent nitrogen; therefore, at least 2.5 mg of protein or 10 mg of dry weight of cells (assuming a conservative value of 25 per cent protein content in cells) are needed for reasonably accurate determination by the standard micro-Kjeldahl technique. The biuret test, on the other hand, can be used as an effective analytical procedure for from 1 to 14 mg protein or a minimum of 4 mg of dry weight of cells. When cell samples smaller than 4 mg of dry weight of cells are to be analyzed, none of the above methods will be applicable.

During present biochemical response studies in the Warburg apparatus it was necessary to determine intracellular protein content for a very small quantity of cells. For example, in typical experiments a substrate

concentration of 500 mg/l was used with a small initial inoculum of cells, giving rise to cell concentrations varying from 10 to 250 mg/l or 0.8 to 10 mg dry weight of solids per Warburg flask (mixed liquor volume in flask = 40 ml). Since a number of analyses such as solids production, cellular carbohydrate and protein content were performed on the contents of each Warburg flask, it was not possible to obtain a cell sample within the requirements of the micro-Kjeldahl or biuret method for protein determination. If it was desired to use either of the above methods for cell protein analysis, drastic changes in experimental protocol would have been necessary and the large amount of data required in this work could not have been obtained. Since only a limited number of flasks could be set on the Warburg apparatus, there was an imperative need for some other more sensitive method for protein assessment.

A very sensitive test for protein analysis using Folin-Ciocalteau phenol reagent was proposed by Wu in 1922 (68). Since then the method has been extensively studied and modified by Lowry et al. for use in general biochemical work (69). Before use in the present study a feasibility investigation was conducted for use of this test in analysis of small amounts of sludge protein.

Folin-phenol reagent is very commonly used in basic research in the biochemical fields. With suitable adaptations, procedures are available for the determination of as

little as 0.2 γ of precipitated protein; however, for general work a range of 30 - 300 micrograms is recommended. The final color development with protein is a product of two distinct reactions: (1) reaction with copper in alkaline solution; (2) reduction of the phosphomolybdic-phosphotungstic reagent (Folin reagent) by the copper treated protein. The color obtained in the absence of copper is solely attributed to tyrosine and tryptophan content of the protein (69)(70). It should be noted that although the alkaline copper reaction is a biuret type reaction, in this test it is not strictly proportional to the number of peptide bonds. Also, the incremental increase in color contributed by the so-called biuret reaction on the addition of copper with Folin's reagent is not constant for all proteins; thus color formation in this test is contributed by both the biuret type reaction and the tyrosine and tryptophan content of the protein. Only a few substances encountered in biological work, such as uric acid, guanine and xanthine, cause serious interference with the test (69)(71). Ammonium ion concentration greater than 410 mg/l decreases the color development (69). For all practical purposes, in sanitary engineering research NH_4^+ ion interference can be safely neglected when analyzing for protein content of filtered sludge.

Reagents

The reagents employed are as follows:

Reagent A - 3 per cent Na_2CO_3

Reagent B - 0.5 per cent sodium tartrate and 0.025 per cent $CuSO_4 \cdot 5 H_2O$

Reagent C - Mix 50 ml reagent A with 1 ml reagent B (maximum life of reagent C is one day)

Reagent D - Dilute Folin reagent (1N). Titrate with standard alkali (NaOH) to a phenolphthalein end point

1 mg/ml of crystalline bovine serum albumin solution may be used as the standard protein.

Protocol

The experimental protocol for the test is as follows: Obtain a sample of cells containing protein between 25-500 µg, by centrifugation or by filtering a known volume of mixed liquor through a Millipore filter. Suspend the sludge sample in 0.5 ml of distilled water. The use of a direct mixed liquor sample is not recommended because of the critical pH considerations, especially when the medium is buffered. Add 0.5 ml of 1N NaOH to the sample and shake the sample intermittently. After thirty minutes add 5 ml of reagent C followed by mixing. The solution is allowed to stand at room temperature for at least ten minutes. Then 0.5 ml of reagent D is added and the contents mixed immediately. Rapid mixing after the addition of Folin reagent is very critical for full development of the color. After

standing thirty minutes the samples are centrifuged and the blue supernatant is read at 750 mμ. A reagent blank and protein standards should be simultaneously run.

Standard Protein

A working standard was prepared from crystalline bovine serum albumin. A standard curve was run using a Beckman Model DU spectrophotometer. A typical standard curve read at 0.3 mm slit width and 750 mμ wave length is shown in Fig. 2. It is seen that the relation between color intensity and the protein concentration is not quite linear. This fact has also been pointed out by Lowry et al. working with rabbit brain protein (69). It is emphasized that the standard curve is highly reproducible but it is recommended that standards be run with each set of samples.

For the biuret test on filtered sludge samples, Gaudy recommended a 15 minute boiling period with 2N NaOH (normality in made-up volume = 2/4.5 = .44N)(65). Since no boiling of cell samples is recommended in the presently discussed method, the effect of heat treatment on the release of protein from cells was studied. Accordingly, a duplicate set of standards and cell samples were prepared. After the addition of 0.5 ml 1N NaOH (normality in made-up vol = 0.5N), one batch of standards and samples was incubated at 60°C. for one hour. The second batch of standards and samples was incubated at room temperature for thirty

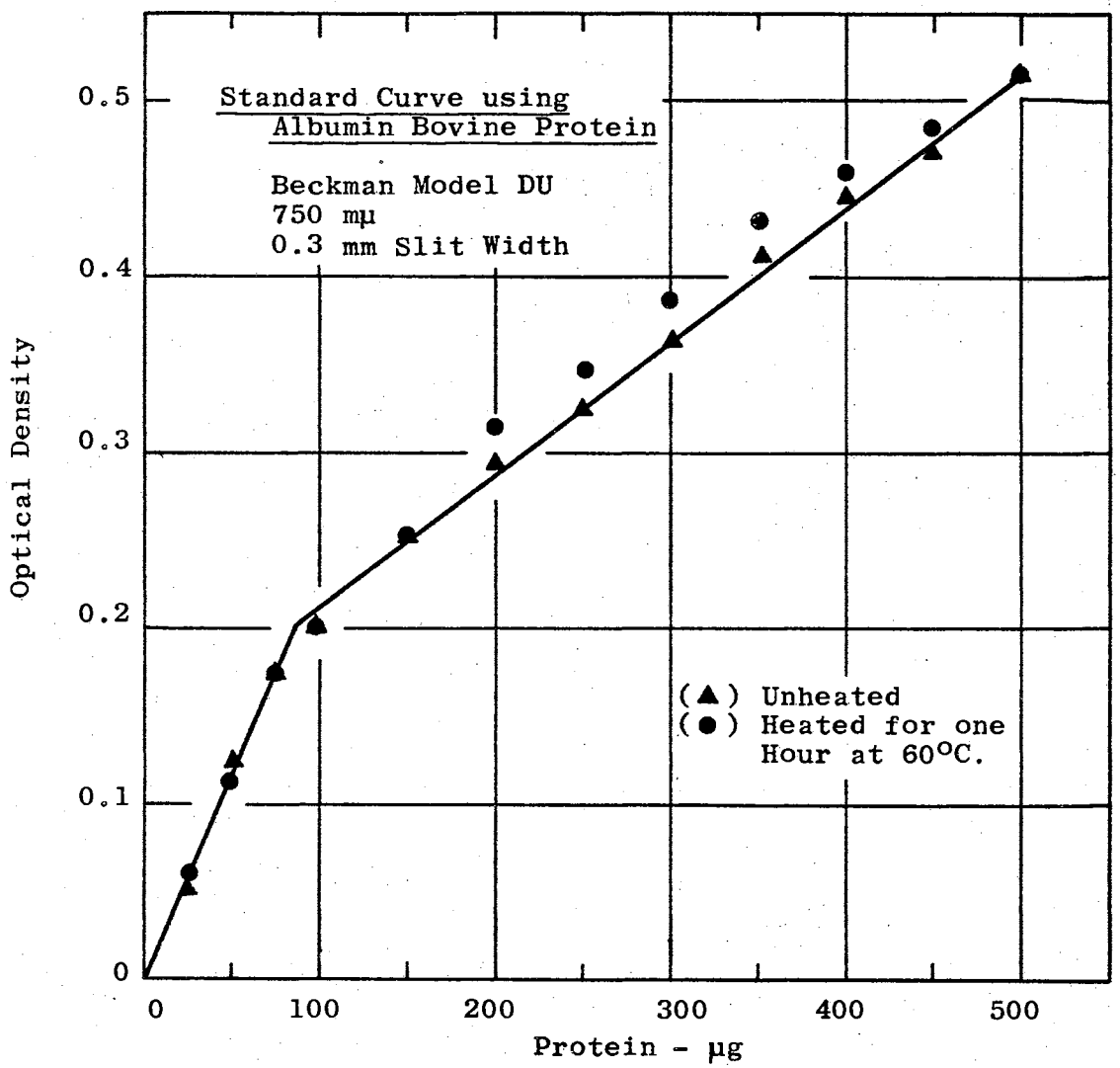


Fig. 2 - COPPER-FOLIN DETERMINATION OF PROTEIN.

minutes. The Copper-Folin test was performed on both batches. The values for the heated standards are shown in Fig. 2. The results for various heated and unheated samples are shown in Table II. The values for heated samples were computed using the unheated standard. In general, lower values were obtained for heated samples. Computations of values using the standard curve would have lowered the results still further. Also, heat treatment did not yield a good standard curve. From this study it was concluded that the recommended treatment was adequate to release protein from the cells.

Centrifugation of the reaction fluid before reading the samples did not have a very significant effect on the final reading. An increase of 1 per cent transmittance was the maximum observed. However, centrifugation of the sample prior to making the reading is recommended.

TABLE II

PROTEIN DETERMINATION OF A BIOMASS USING COPPER-FOLIN TECHNIQUE

Sample No.	Protein Content, mg/l	
	Unheated	Heated for 1 hr. at 60°C.*
1	88	77
2	75	75
3	92	78
4	180	163
5	217	195
6	235	215

*Values are computed based on unheated standard

Conclusions pertaining to the Copper Folin Method

The measurement of protein with copper and Folin reagent has a major advantage when very small cell samples are available and especially when other methods for protein analysis cannot be used due to lack of adequate quantity of samples. The method possesses all of the advantages in experimental technique of the biuret test, and is less cumbersome than the Kjeldahl technique. However, two disadvantages of the Copper-Folin reaction should be fully realized: (1) the amount of color varies with different proteins, and in this regard the biuret test is more desirable; (2) the color is not strictly proportional to concentrations of protein over a wide range. Considering all the advantages and disadvantages, the most reasonable application of the Copper-Folin reaction would be in cases when absolute values of protein are not needed and when the sample size is too small to be analyzed by the biuret method.

Protein Determination - Biuret Method

When sufficient quantities of cell samples were available, the biuret method for protein analysis was employed. The protocol used was exactly as described by Gaudy (65).

Experimental Protocols

An attempt is made here to cover the salient features of each phase of the study. Specific details of protocol for some experiments are given as the results are presented.

PHASE I - Investigations into Causation of the Plateau in Mixed Population Systems

A. Seed Preparations

The following seed preparations were used:

1. Sewage Seed

Supernatant from settled sewage obtained from the effluent of the primary clarifier of the municipal sewage treatment plant at Stillwater, Oklahoma, was used as seeding material and was designated as sewage seed.

2. Acclimated Seed

Acclimated populations were obtained by placing 2 ml "sewage seed" in 60 ml of synthetic medium (Table I) containing 3000 mg/l of glucose. This system was aerated on a laboratory shaker at 100 strokes per minute and temperature maintained at 25°C. After 24 hours one ml was transferred into fresh medium. Three serial transfers were made on successive days, and on the fourth day of operation cells were harvested near the end of the log growth phase (after approximately 18 hours of aeration) by centrifugation, washed twice in 0.05M phosphate buffer pH 7, and used for experimentation.

3. Pasteurized Seed

Ten ml of the seed in question was incubated for five minutes at 50°C. (122°F.). The seed was cooled to room temperature and used in the experiment.

4. Filtered Sewage Seed

Ten ml of the settled sewage was run through the desired filter paper (Whatman No. 1 or Millipore filters of pore sizes 1.2μ, 3μ, 5μ, or 10μ), and employed in the experiment.

5. Diluted Seed

This seed was prepared to obtain various protozoa-bacteria ratios in the initial inoculum. This was achieved by altering the initial protozoa populations in the seed. The following formula was used in the preparation of the diluted seed:

S_x = 2 ml of pasteurized S + 2 ml of S diluted x/2 times with 0.05 phosphate buffer pH 7

where

S - sewage seed

x - protozoa dilution factor with respect to the original seed, which in this case is the sewage seed.

6. Protozoa-rich Seed

Two ml of settled sewage were placed in 60 ml of synthetic medium (Table I) containing 500 mg/l of glucose. This system was aerated on a laboratory shaker at 100 strokes per minute for 48 hours. The temperature was maintained at 25°C. An inoculum from this mixed liquor was designated as a "protozoa-rich inoculum" or "protozoa-rich seed". Preliminary experimentation indicated that this

procedure provided a seed with a fairly high mumber of protozoa.

B. High Energy Systems - Warburg Studies

For Warburg studies a batch of inorganic salt medium (Table I) was made up and appropriate amount of stock glucose solution was added to yield a desired concentration in the medium. Forty ml samples were then placed in the Warburg flasks and the system was equilibrated for fifteen minutes before closing the manometers. Readings were made at short intervals (as small as 15 minutes) to define closely the oxygen uptake curve. On the average about 40 readings for oxygen uptake were taken for experiments run for 120 hours. The points are not plotted on the curves shown in the Results section simply because they were too numerous. When bacteria or protozoa counts were recorded, one flask was taken off at each sampling point. The contents of the Warburg vessel were thoroughly homogenized by repeated aspiration with a sterile pipet. The samples were then appropriately diluted, and counts were made.

For the study of biochemical parameters such as substrate removal by the COD and anthrone test, intracellular protein, cellular carbohydrate or biological solids, a flask was removed at each sampling point. The solids adhering to the inside wall of the flask were suspended in the reaction mixture, and the contents of the

flask were well homogenized. Twenty ml of the flask contents were passed through a tared membrane filter for the biological solids determination. The filtrate was used to determine the COD remaining in the system. In order to obtain a cell sample for cellular protein and carbohydrate determination an additional 15 ml of mixed liquor from the flask was filtered through an untared membrane filter. The cell mat was completely removed with a spatula and was resuspended in two ml of distilled water. This cell suspension and 5 - 10 ml of filtrate were immediately stored in a freezer for determination of protein and carbohydrate content at a later date. In some experiments flasks were immediately chilled in an ice bath after removal from the Warburg apparatus in order to slow down metabolism. This was done to minimize errors in experiments for which cells were not flocculated, thus increasing the time to filter the sample.

C. Low Energy Systems - BOD Studies

Required amounts of deionized water were sterilized in ten-liter Pyrex carboys. The water was cooled and equilibrated for 48 hours at 20^{o}C. Thirty minutes before the beginning of an experiment the water in each carboy was aerated by aspiration, using a porous diffuser. Appropriate concentrations of inorganic salts, seed material and substrate were then added to the carboys. Substrate was withheld from seed blanks. After thorough mixing, the

bottles were filled by a slow syphoning procedure in order to avoid any air entrainment in the BOD bottles. The bottles were then transferred to a 20°C. incubator. At each sampling time, prior to the dissolved oxygen determination, one ml of sample was withdrawn from the bottle (with a one ml sterile pipet) for viable bacterial cell count. When protozoa were also counted, two BOD bottles were removed at each sampling time. One bottle was used for dissolved oxygen determination, while the other was used for bacteria and protozoa counts. The $Na_2S_2O_3$ titrant was standardized approximately every third day.

D. Studies on the Interaction of Bacteria and Protozoa

These studies were carried out in both high and low energy systems.

1. High Energy System - Warburg Studies on Mixed Bacteria Cultures

Synthetic waste (Table I) containing 500 mg/l of glucose and a small inoculum of pasteurized acclimated seed was placed in four Warburg flasks fitted with sidearms, and oxygen uptake was followed. At the end of 24 hours three flasks were inoculated with one ml of "protozoa-rich seed" via sidearms. The fourth flask was maintained as a control and was inoculated with one ml of the inorganic salt medium (Table I). One flask was removed at each selected sampling time, and protozoa count was recorded. A pasteurized

acclimated seed blank was inoculated similarly with one ml of "protozoa-rich seed" and oxygen uptake was recorded.

2. Low Energy System - BOD Studies on Mixed Bacterial Cultures

A large number of BOD bottles were set up using pasteurized sewage seed and a glucose concentration of 8 mg/l; seed blanks were prepared simultaneously. After 48 hours of incubation at 20°C. one batch of BOD bottles was inoculated with one ml of "protozoa-rich seed" per bottle. A second batch was inoculated with one ml of a 1/10 dilution of the "protozoa-rich seed." In addition, seed blanks were also inoculated with one ml of "protozoa-rich seed." The bottles were carefully restoppered to avoid trapping of air. Samples were removed at selected intervals, and dissolved oxygen and bacteria and protozoa counts were determined for each sample.

3. Interaction between a Pure Culture of Bacteria and Protozoa - Warburg Studies

(a) Serratia marcescens as Food for Protozoa

A pure culture of Serratia marcescens supplied by the microbiology department of Oklahoma State University was used as a substrate for protozoa in this study. The pure culture was grown in 3000 mg/l glucose synthetic waste (Table I) using NH_4^+ as nitrogen source. The tech-

nique for culture enrichment similar to that described for "acclimated seed" earlier in this chapter, was employed. After three serial transfers (24 hours of incubation each), pure culture cells were harvested by centrifugation, washed twice in 0.05 M phosphate buffer pH 7 and were resuspended in inorganic salt medium (Table I) containing $(NH_4)_2\ SO_4$. The pure culture suspension was split into two 100 ml portions. One portion was inoculated with one ml of "protozoa-rich seed". Duplicate Warburg flasks were set up for both systems. One flask in each system was used for obtaining bacteria and protozoa count data, while others were used to record oxygen uptake. An endogenous blank for the protozoa alone was also maintained.

(b) Varying Concentrations of Serratia marcescens as Food for Protozoa

In this experiment the pure culture cell suspension was obtained in the manner described above (3,a). Three different cell concentration systems consisting of controls and flasks inoculated with protozoa were set up on the Warburg apparatus. Two ml of "protozoa-rich seed" was used per 100 ml of cell suspension.

E. Effect of Chloramphenicol

1. Effect on Growth and Protein Synthesis

A two-liter batch activated sludge unit was started with 2 per cent sewage seed using the inorganic salt medium

given in Table I, and 1000 mg/l glucose as sole carbon source. The system was aerated and sampling was begun when turbidity in the medium was discernible. When the solids concentration in the system was nearly 300 mg/l as judged by optical density, the mixed liquor volume was split into three batch units. Chloramphenicol stock solution was added to two units to obtain concentrations of 10 mg/l and 50 mg/l respectively. The third unit was followed as a control. In each of the three systems the volume of reaction liquor was 0.5 liters. The units were sampled at one-hour intervals. Solids determinations were made using 25 - 40 ml samples of mixed liquor. For cell protein determination the solids in 15 to 40 ml samples were retained on a 0.45 μ Millipore filter and resuspended in 4 ml of distilled water. This concentrated cell suspension was used for protein analysis. Viable cell count was also estimated.

2. Effect on Protozoa

A one liter batch activated sludge unit was started with one per cent sewage seed and 1000 mg/l glucose using the synthetic medium shown in Table I. The reaction mixture was examined intermittently for protozoa and sampling for protozoa count was begun as soon as the protozoa count could be recorded in the system. Four 50 ml portions of mixed liquor were transferred to 250 ml Ehrlenmeyer flasks and aerated on a laboratory shaker at 100 oscillations per minute. Appropriate amounts of chloramphenicol were added

into the flasks to attain concentrations of approximately 50, 100, 200 and 500 mg/l respectively. The bacterial count in the system at the time of treatment with chloramphenicol was recorded. Protozoa counts in the systems were followed throughout the study.

3. Effect on Oxygen Uptake

Four Warburg flasks (fitted with sidearms) holding 39 ml of salt medium (Table I) containing 540 mg/l glucose and 0.5 per cent sewage seed were set up. Into the sidearm of one flask one ml of chloramphenicol at 2000 mg/l was placed; another flask received one ml of chloramphenicol at 400 mg/l. One ml of distilled water was placed in the remaining two control flask sidearms. The systems were placed on the Warburg apparatus, and oxygen uptake was followed. The contents of the sidearms were tipped into the vessels when 190 mg/l of accumulated oxygen uptake was registered in each flask. This value was chosen because it was close to the end of the first stage and well before the initiation of the second stage of oxygen uptake. Simultaneously one control flask was removed for analysis of substrate by the anthrone and COD tests at the time of tipping the inhibitor into the flasks. The biological solids produced were also determined.

PHASE II - Investigations into the Causation of the Plateau in Pure Culture Systems

A. Sources of Pure Cultures

1. Previously Identified Organisms

Identified pure cultures were obtained from the department of microbiology at Oklahoma State University.

2. Warburg Experiments

Pure cultures were isolated from the Warburg experiments, using heterogeneous populations in Phase I, which exhibited a typical diphasic oxygen uptake expression with two phases separated by a plateau.

3. Activated Sludge Units

Pure cultures were obtained from one liter batch activated sludge units started with sewage seed. These units were fed daily 1000 mg/l glucose or 1000 mg/l phthalic acid using standard synthetic waste (Table I). No wasting of mixed liquor was practiced. The units were maintained for 2 - 3 days after which they were restarted from fresh sewage. Bacteria were isolated from these systems.

B. Isolation Procedure

Nutrient agar (Difco) was used as a culturing medium for the purpose of isolating pure cultures. Successive subculture streaks were made to ensure the purity of a culture. Pure cultures were stored on nutrient agar slants.

C. Oxygen Uptake Studies

The pure culture inocula were prepared in a manner similar to that described for "acclimated seed" in Phase I, using 3000 mg/l of substrate. In the case of phthalic acid substrate, the pH of the 5000 mg/l phthalic acid stock solution was adjusted to 7 with 20 per cent KOH. For oxygen uptake measurements cells were not washed but a one ml inoculum of the culture was used per 100 ml of experimental salt medium (Table I) containing 500 mg/l of substrate; therefore substrate carryover was very slight.

D. Extended Study of Pure Culture No. 13

1. Substrate Concentrations vs Oxygen Uptake

Experimental protocol similar to that described above (C) was employed except that glucose concentrations of 100 - 900 mg/l were used for oxygen uptake measurements. An endogenous blank was also run.

2. Initial Biological Solids Concentrations vs Oxygen Uptake

Adequate amounts of cells were grown on a laboratory shaker using the operational procedures described for "acclimated seed". Cells were washed once with 0.05M phosphate buffer pH 7 before use. Systems containing various initial solids concentrations were set up using 500 mg/l of glucose as the carbon source. Endogenous controls were also run for each system.

3. Oxygen Uptake on Various Substrates

Oxygen uptake on the Warburg apparatus was measured for different substrates using small inocula of glucose-grown cells and cells acclimated to the respective substrates. The following substrates were used:

(a) Sterile municipal sewage
(b) Nutrient broth solution (Difco Labs)
(c) Sodium acetate (J. T. Baker Chemical Co.)
(d) Sodium glutamate, Mono (Matheson, Coleman and Bell)
(e) d-Sorbitol (Fisher Scientific Co.)
(f) D-(-)-Ribose (Eastman Organic Chemicals)

4. Inhibitor Studies

Seven Warburg flasks (with sidearms) were set up containing 540 mg/l of glucose in inorganic salts medium (Table I). A small inoculum of pure culture No. 13 which had been washed twice was used as seed. One ml of 2000 mg/l chloramphenicol solution was placed in the sidearms of each of four flasks. The remaining three flasks were followed as controls. Chloramphenicol was tipped into the reaction mixture at various times as desired. One control was removed at the desired time to determine substrate concentration by the anthrone test. Bacteria counts were run on a control flask and another flask into which inhibitor was tipped at 10.5 hours after the beginning of the experiment. For this particular system duplicate flasks were run. One was used for oxygen uptake, and the second for the viable bacteria count.

5. Biochemical Response Studies

The experimental protocol described for mixed population "Warburg Studies" was used. When volatile acids were determined, two to three flasks were taken off and a sample of 60 - 88 ml of filtrate was used for immediate volatile acids determinations. In addition, carbohydrate analyses were made on the cells.

6. Endogenous Metabolism Studies

The desired amount of pure culture No. 13 was cultivated in 3000 mg/l glucose and salt medium (Table I) on a laboratory shaker. A complete 4-day acclimation procedure (described under "Acclimated Seed" Phase I) was followed. The cells were harvested on the fourth day (after 18 hours of incubation on the shaker), and were washed once with 0.05 M phosphate buffer, pH 7, before use in the experiment. The washed cells were suspended in salt medium (Table I), and the required number of Warburg flasks were set up. The course of oxygen uptake was recorded. Samples were taken by removing Warburg flasks at appropriate intervals. COD and anthrone analyses were made on the filtrate. Also, the protein (biuret method) and carbohydrate contents of the cells were determined. The COD values reported were corrected for the COD of the medium without the organic substrate.

PHASE III - Phasic Oxygen Uptake in Multicomponent Substrate System

Glucose-or sorbitol-acclimated seed was grown on the shaker using NH_4^+ nitrogen and the standard salt medium (Table I). BOD bottles were set up using the desired seed, glucose, and sorbitol concentrations. Two BOD bottles were removed at each sampling point. One bottle was used for determination of dissolved oxygen. The contents of the second bottle were filtered through a membrane filter (HA, 0.45μ) and 200 ml of filtrate was concentrated to 5 ml using the flash evaporator. These 5 ml samples were frozen for later analyses of substrate.

CHAPTER V

RESULTS

PHASE I - Investigation into Causation of the Plateau in Mixed Population Systems

Investigations Related to Theory 1

A. A Preliminary Study

An exploratory study was made to determine the possible role of protozoa in the expression of oxygen uptake. Oxygen uptake curves using glucose synthetic waste and various types of seed are shown in Fig. 3. The typical diphasic kinetics of oxygen uptake, separated by a plateau, were expressed using sewage seed. While the pasteurization of seed prevented the occurrence of the second phase of oxygen uptake, the filtration of seed (through Whatman No. 1 filter paper) enhanced a well defined separation of the two phases of oxygen uptake. The filtration of seed also caused the second stage of oxygen uptake to diminish as compared to the sewage seed system. A slight rise in oxygen uptake was observed after 80 hours in the pasteurized seed system.

The results of this preliminary experiment were interpreted as follows: The protozoa contribute to a large

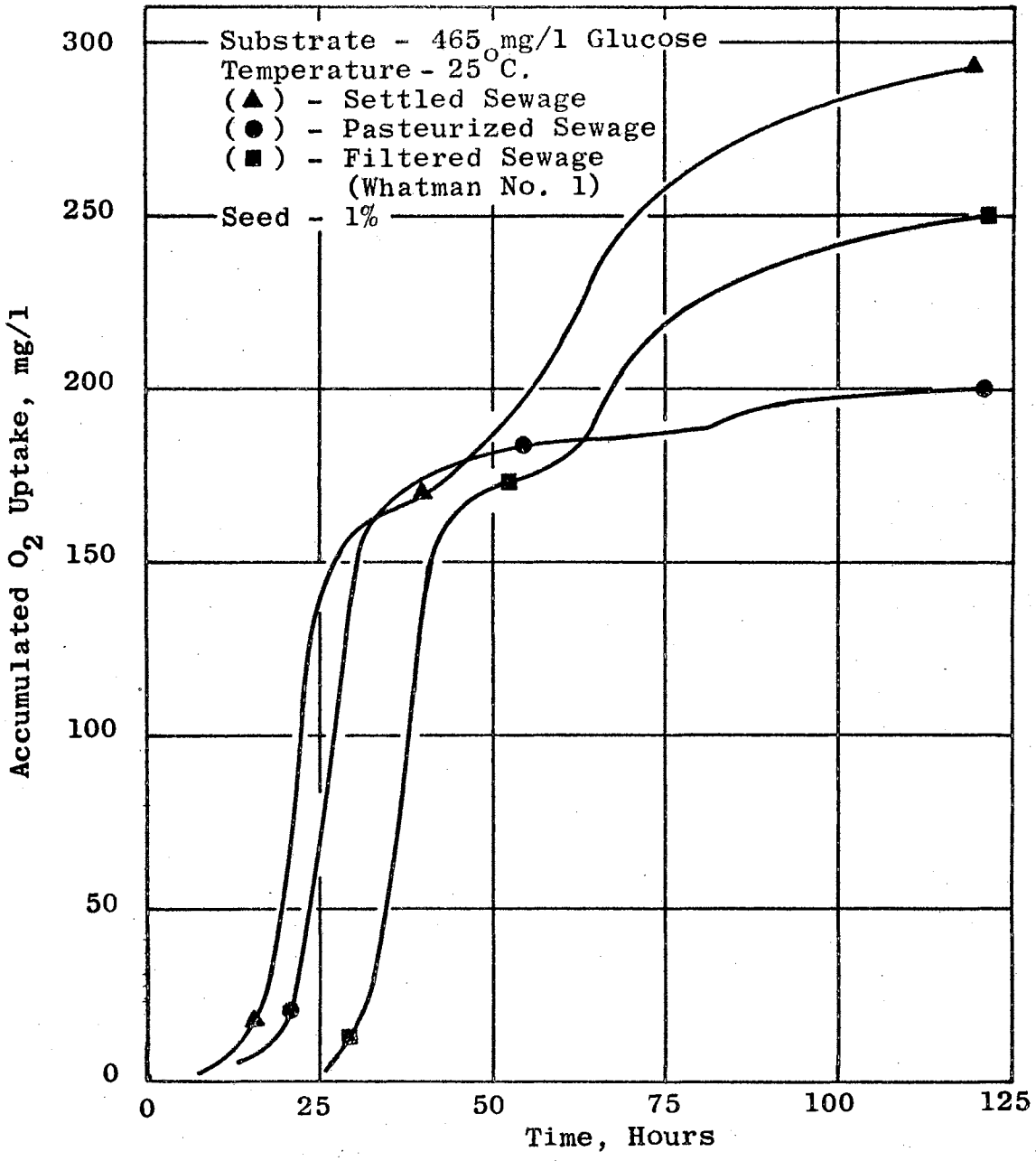


Fig. 3. WARBURG STUDIES USING VARIOUSLY TREATED SEWAGE SEED.

extent to the secondary stage of oxygen uptake. The small secondary rise in O_2 uptake noticed in the pasteurized seed system appeared to be due to the germination of protozoan cysts which survived the pasteurization procedure. The modification in the oxygen uptake curve due to filtration of the seed probably resulted from the combined effect of initial reduction in number of protozoa and selection in initial protozoa populations in the seed. However, the possibility that these modifications in oxygen uptake were due to a possible bacterial selection, through pasteurization and filtration treatment, was not entirely eliminated.

B. Filtration Studies

In order to delineate further the extent of participation of protozoa in the secondary stage of oxygen uptake, a more defined method for physical removal of protozoa from the seed was adopted. Protozoa are finitely larger than bacteria. The minutest kinds of protozoa known are a wide variety of flagellates belonging to the Monadidae family ranging in length from 2 to 10 μ. Thus microfiltration of seed was a suitable procedure for excluding protozoa from the seed without causing a restrictive selection in the bacterial population, particularly species which might have been eliminated by exposure to heat during the pasteurization procedure.

Figures 4 and 5 show plots of oxygen uptake for two typical runs using sewage seed and sewage seed filtered through Millipore filters of various sizes (1.2, 3, 5, and 10 microns). In addition, in Fig. 5 the plots of oxygen uptake for pasteurized seed are also shown. These two experiments were run at different times. Oxygen utilization (Figures 4 and 5) for both the unfiltered system and the one filtered through a 10 μ filter exhibited two distinct phases. However, the separation of two stages was more emphasized when 1 per cent seed (Fig. 4) was used as compared to the 2 per cent sewage seed system (Fig. 5). It can be seen that there was no significant effect of the straining of seed through the 10 μ Millipore filter on the oxygen absorption pattern as compared to the untreated sewage seed. No second phase of oxygen uptake was observed for 5 μ filtered seed (Fig. 5), but the same system in Fig. 4 showed a delayed and very small second stage, whereas the second stage of oxygen consumption was completely absent for systems employing seed inoculum filtered through 1.2 and 3 μ filters. Total oxygen utilization in the 1.2, 3 and 5 μ filtered systems in Fig. 5 was considerably higher than in the pasteurized seed system, although the former did not show any second stage of oxygen uptake. Long lag periods in O_2 uptake were noted in the systems in which the seed was filtered through the finer pore size filters.

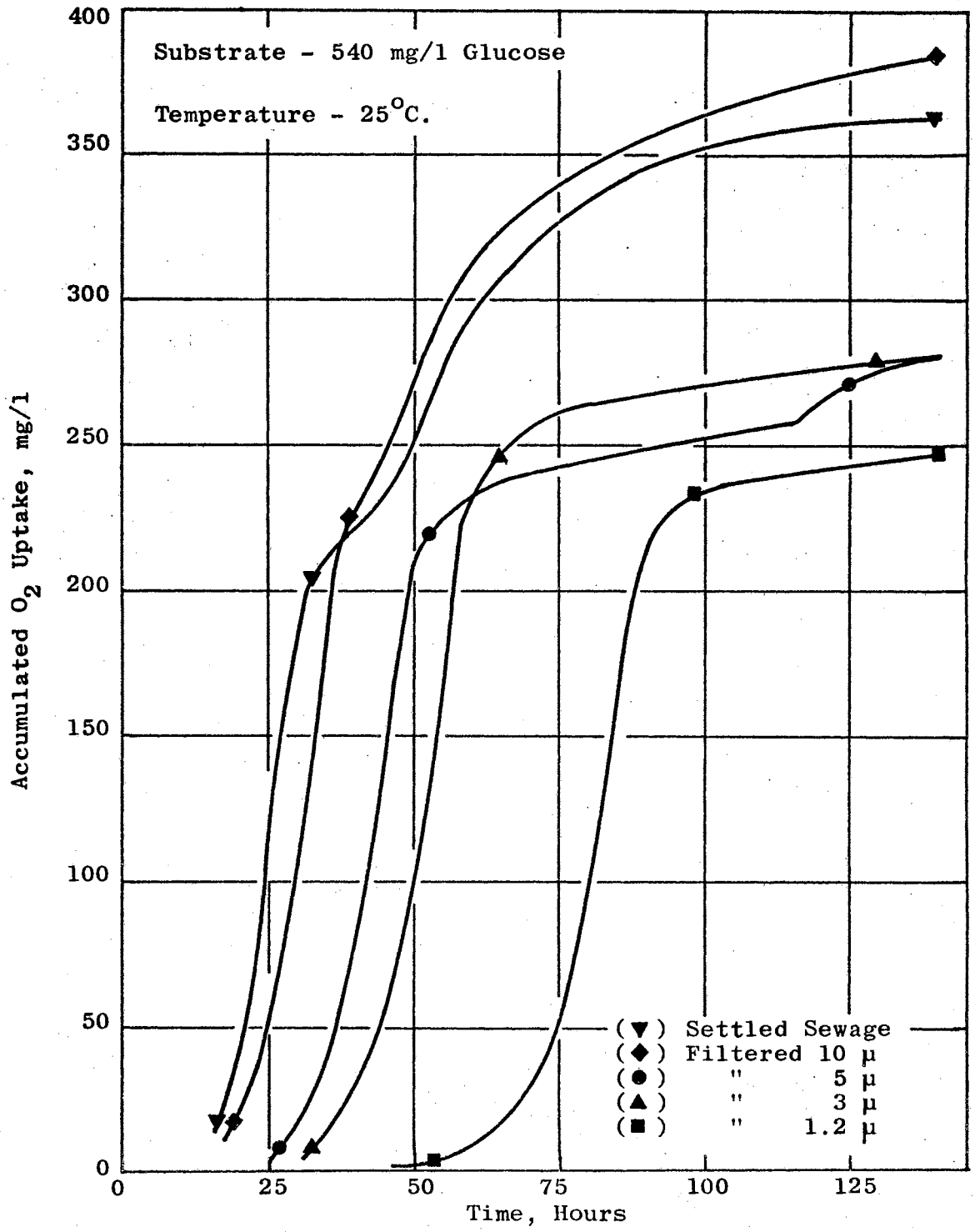


Fig. 4. EFFECT OF FILTERING SEWAGE SEED THROUGH VARIOUS SIZE MILLIPORE FILTERS ON O_2 UPTAKE USING MANOMETRIC TECHNIQUE - 1% SEWAGE SEED

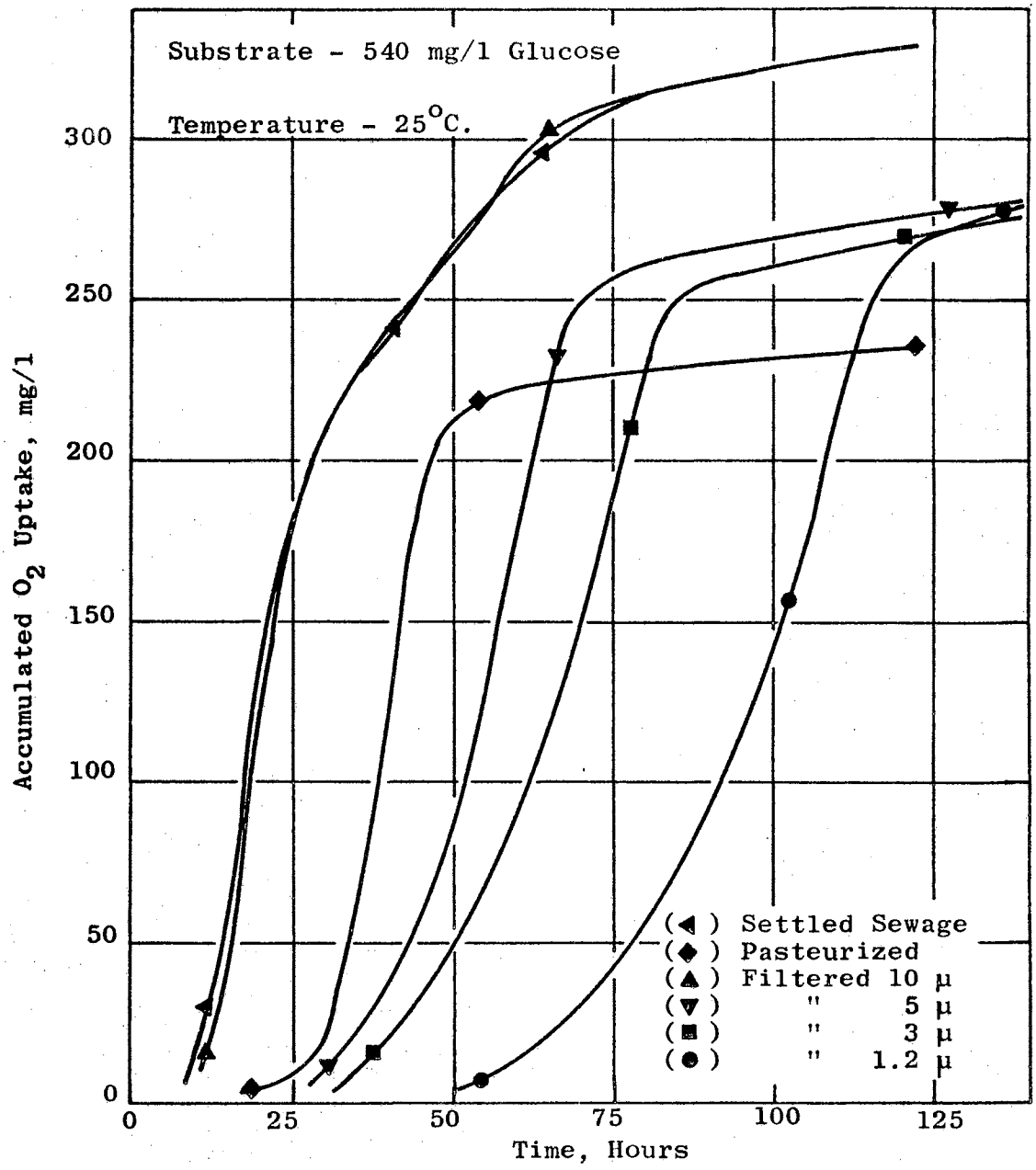


Fig. 5 - EFFECT OF FILTERING SEWAGE SEED THROUGH VARIOUS SIZE MILLIPORE FILTERS ON O_2 UPTAKE USING MANOMETRIC TECHNIQUE - 2% SEWAGE SEED.

Microscopic examination was carried out on the contents of all flasks at the termination of the experiments. Highly motile, flagellated protozoa were observed in the sewage seed and the 10 μ filtered seed flasks, while less motility and relatively fewer protozoa were observed in the 5 μ filtered seed system (Fig. 4). Absolutely no protozoan activity was noticed in any of the remaining flasks. It was very hard to identify, readily, the kinds of protozoa due to their small size. It was also observed that a higher degree of clarification existed in flasks showing a second stage of oxygen uptake than in those which did not exhibit a secondary oxygen consumption.

The results thus far presented strongly suggested that the protozoa were related to the exertion of the second stage of O_2 uptake, and that most of the protozoan populations responsible for the second stage of oxygen utilization in the sewage seed could be retained on the 5 micron filter but passed the 10 micron filter. Some protozoa which passed 5 μ Millipore filter were also present, but their contribution to the second stage of oxygen uptake was not considerable.

C. Bacterial and Protozoan Population Changes during Oxygen Uptake in High and Low Energy Systems

Fig. 6 shows variations in the numbers of bacteria and protozoa during oxygen utilization on the Warburg apparatus

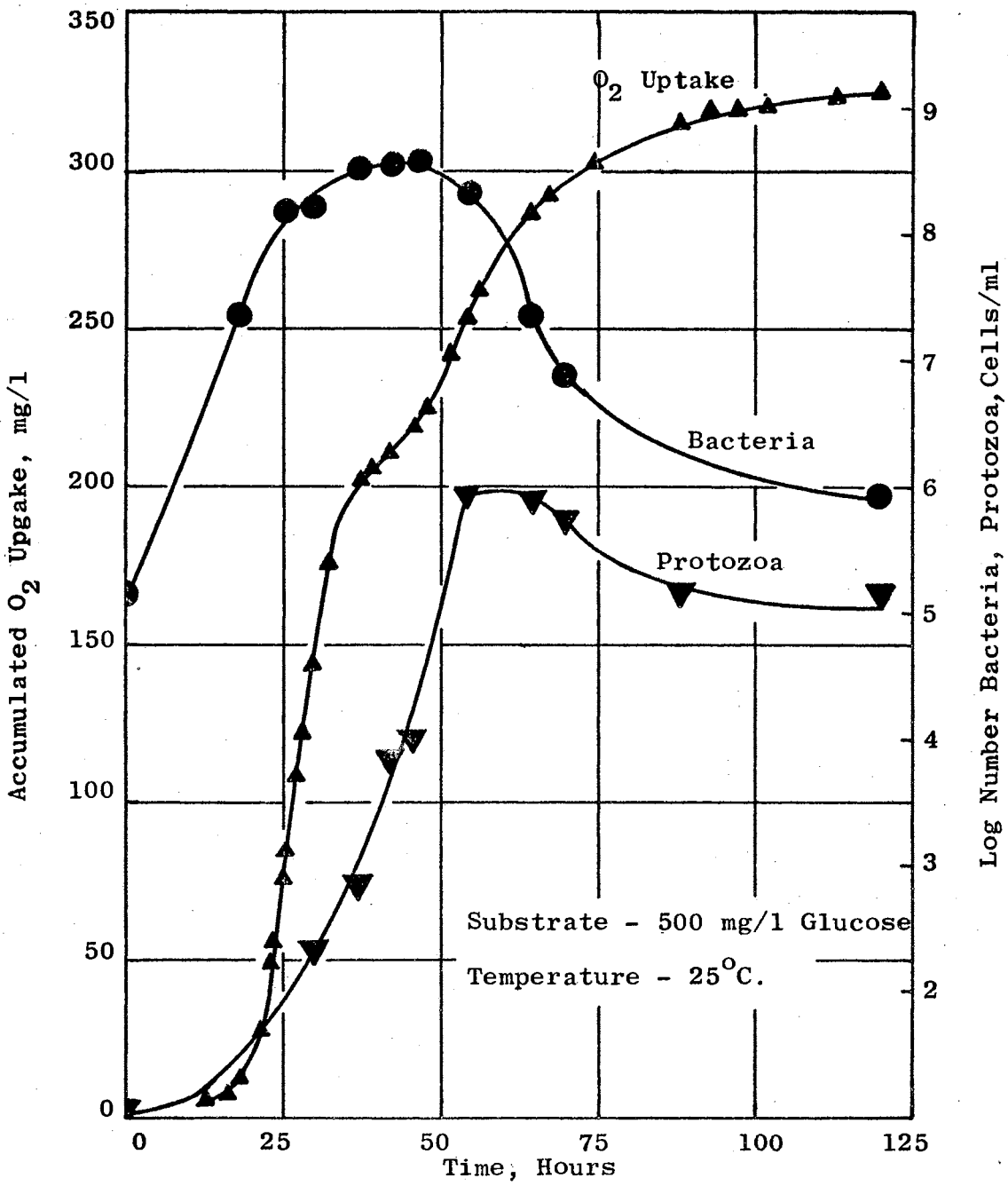


Fig. 6 - BACTERIA AND PROTOZOA COUNTS DURING WARBURG STUDY, USING 2% SEWAGE SEED.

using glucose synthetic waste inoculated with 2 per cent sewage seed. The bacterial population attained its maximum density at a time corresponding to the break in the oxygen uptake curve, and died off rapidly during the second phase of oxygen uptake. Practically no stationary phase was observed in the range of maximum bacterial population, and correspondingly only a small pause was observed in oxygen uptake. The second stage of oxygen utilization was marked by rapidly multiplying protozoa (approximately 7000 cells/ml at the beginning of the second stage of O_2 uptake), and rapidly declining viable bacteria count. The attainment of the maximum protozoan population lagged the bacterial peak by approximately 15 hours, and by the time the protozoa count reached its maximum roughly half of the secondary oxygen demand was already satisfied. In the later part of the experiment both protozoa and viable bacteria decreased in the system.

Microscopic examination showed that the protozoa were mainly flagellates belonging to the genus Monas. A comparatively small number of ciliates were also observed. At least two types were identified as Enchelys and Colipidium.

Fig. 7 shows the oxygen uptake curve and the bacteria count using 2 per cent pasteurized sewage seed. The same sewage seed was used as for the experiment shown in Fig. 6. A second phase of oxygen utilization was not exhibited, and the amount of oxygen uptake more or less corresponded to

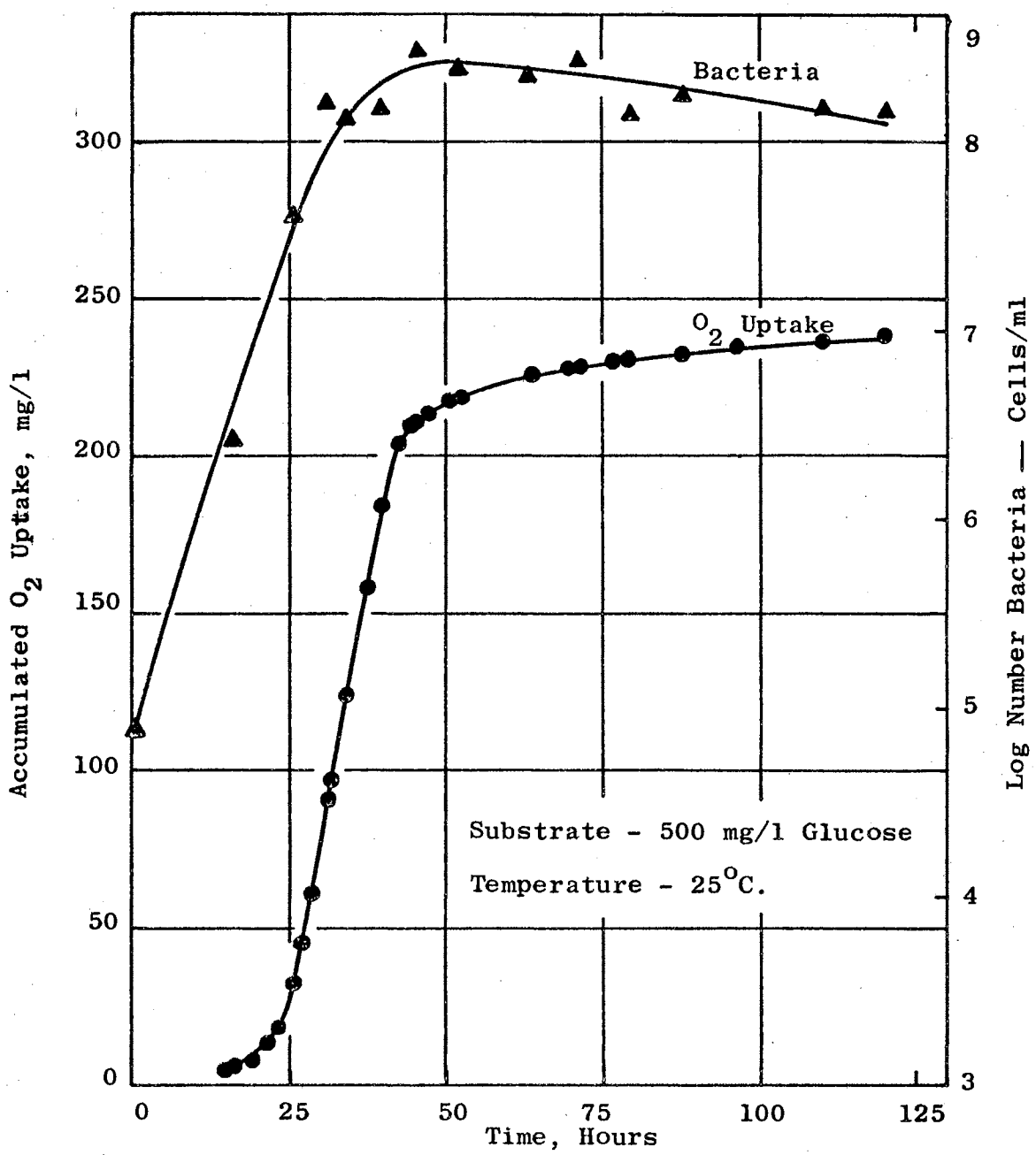


Fig. 7 - BACTERIA COUNT DURING WARBURG STUDY, USING 2% PASTEURIZED SEWAGE SEED.

the first stage of oxygen utilization in Fig. 6. The highest bacterial count was again reached at a time corresponding to the end of the rapid O_2 uptake, and there was only a slight decrease in the viable population. No active protozoa were observed throughout the duration of the experiment. A higher degree of clarification was noted at the termination of the experiment for the sewage seed system (Fig. 6) than in the pasteurized seed system of Fig. 7.

Changes in numbers of bacteria and protozoa during BOD exertion (low energy system) using the sewage and the pasteurized sewage seed are shown in Fig. 8. Again the beginning of the second oxygen uptake phase was accompanied by a rapid increase in protozoa population and a concomitant loss in the viable bacteria population. The peak in numbers of protozoa lagged the maximum bacterial count. A well-defined plateau was generated in the oxygen uptake curve corresponding to the range of maximum bacterial density. Also, fifty percent of the secondary oxygen uptake was exerted at the time of maximum protozoa density in the system. Small, not readily identifiable protozoa were observed.

A phasic BOD curve was not in evidence for the pasteurized seed system and there was no decrease in the viable bacterial population after attaining the maximum count. Although a lag time of about one day was observed in the oxygen uptake and the bacterial population as compared to the sewage seed, the same maximum bacterial density was

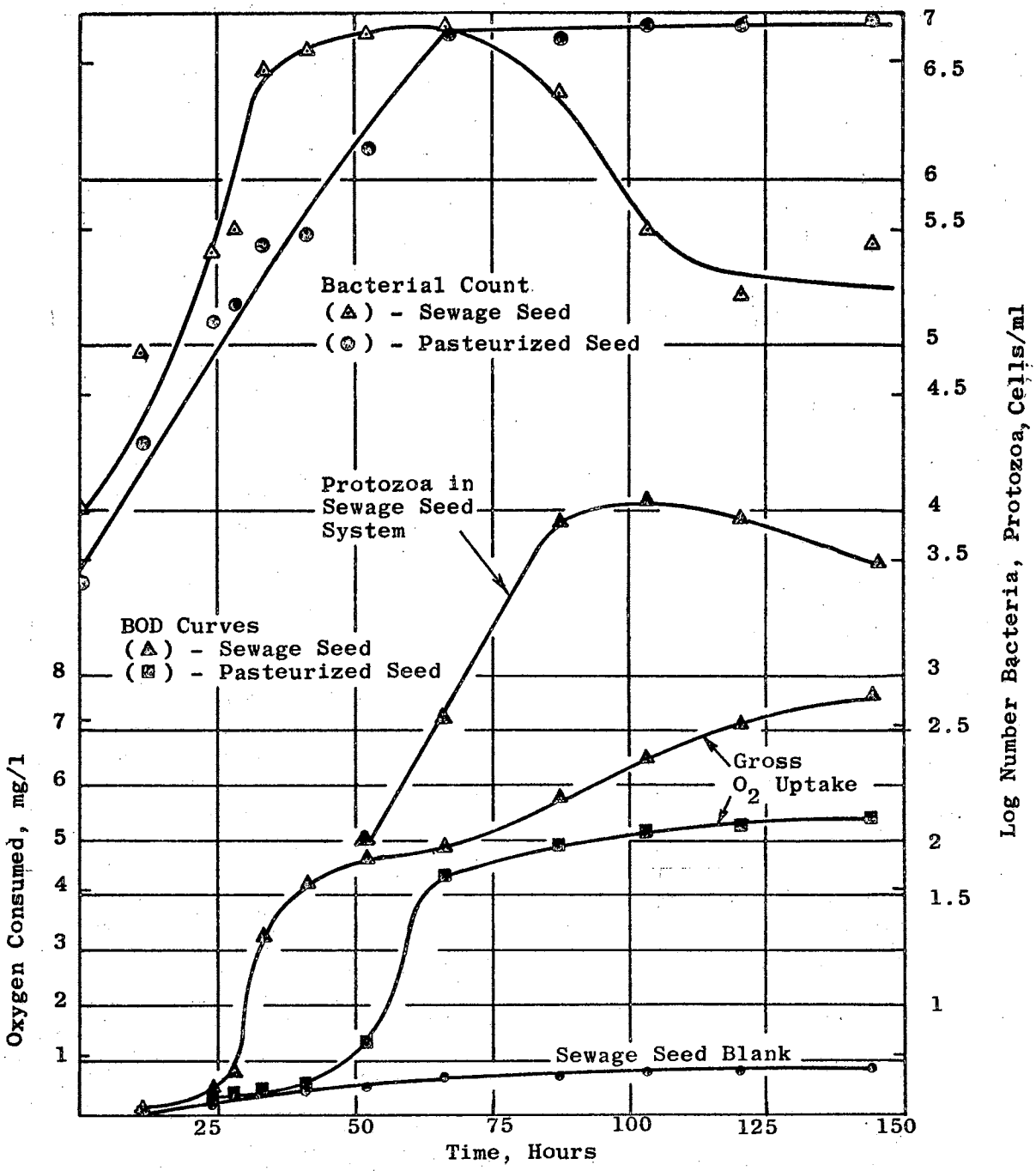


Fig. 8 - BOD EXERTION, BACTERIA AND PROTOZOA COUNTS IN THE BOD BOTTLE, USING 11 mg/l GLUCOSE.

attained in both systems. Also, oxygen consumed in the pasteurized seed system was almost equal to the first stage of oxygen utilization in the sewage seed system. No protozoa were observed in the pasteurized seed system. The sewage seed blank consumed only a small amount of oxygen.

The results of two Warburg experiments using seed acclimated to glucose are shown in Figures 9 and 10. These experiments were run at different times. While the oxygen uptake curve in Fig. 9 showed no discernible diphasic nature, two oxygen uptake phases distinctly separated by a plateau were observed in Fig. 10. A remarkable contrast was noted between the relative kinetics of growth of the bacteria and the protozoa in the two systems. In Fig. 9 the maximum numbers of the bacteria and protozoa were achieved simultaneously; however, a long lag of nearly 30 hours was observed between the peaks of bacterial and protozoan counts in Fig. 10. For some reason the protozoan activity rapidly decreased in Fig. 9 after attaining a maximum, and a loss of only 5.25×10^8 viable bacterial cells/ml was registered at the end of the experiment. Considerably less oxygen demand was exerted in this system at the termination of the experiment (Fig. 9) as compared to that shown in Fig. 10, which showed, also, a greater decrease in the bacterial population. As noted earlier, the plateau in Fig. 10 corresponded to the range of maximum viable bacterial cells/ml. The stationary phase was very extended in this case (over 25 hours).

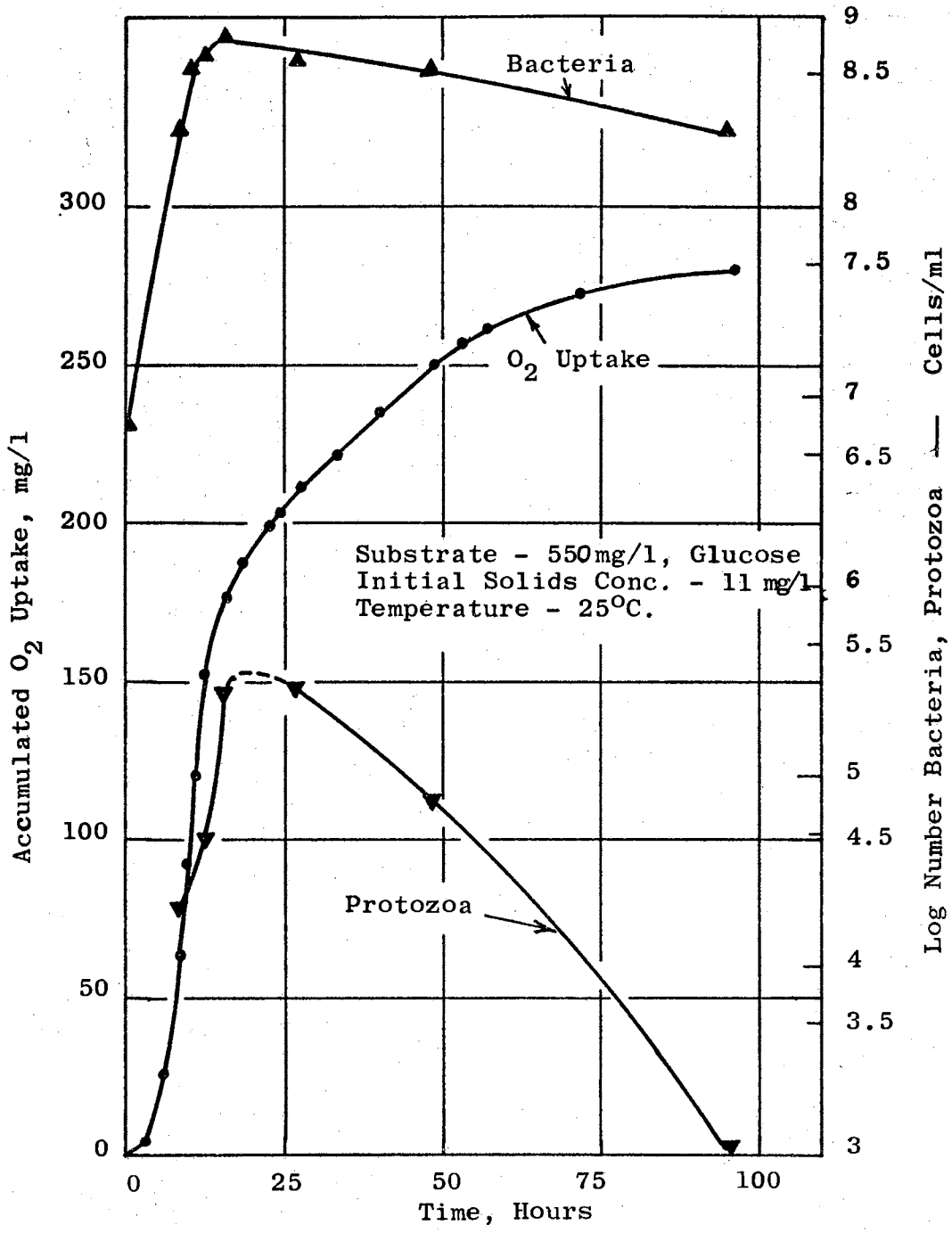


Fig. 9 - BACTERIA AND PROTOZOA COUNTS DURING WARBURG STUDY, USING ACCLIMATED SEED

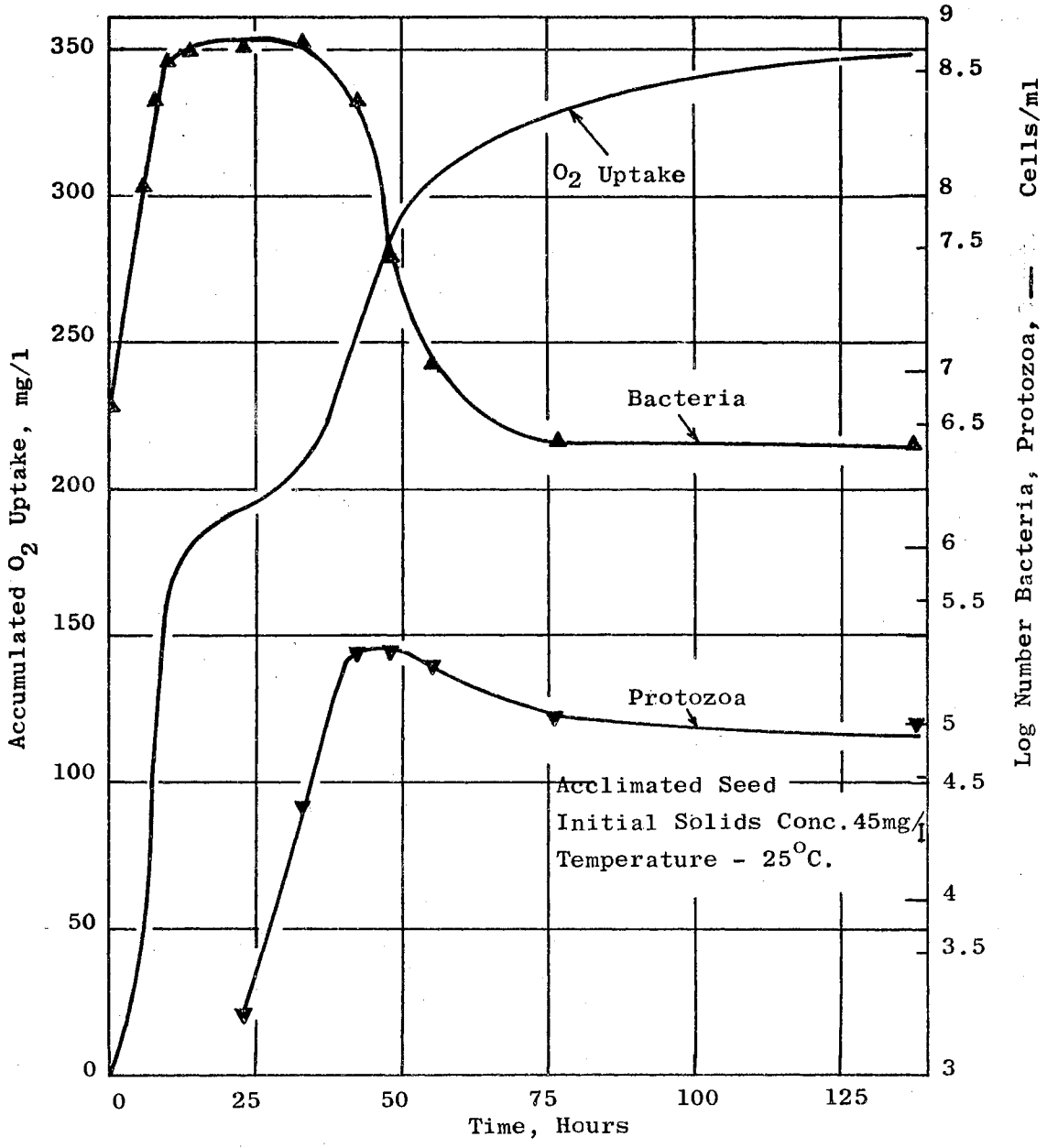


Fig. 10 - BACTERIA AND PROTOZOA COUNTS DURING WARBURG STUDY, USING 600 mg/l OF GLUCOSE

A typical BOD experiment in which substrate removal was assessed is shown in Fig. 11. In this experiment sewage seed was employed. The protozoa appeared quite late in the experiment and showed a rather slow development toward maximum population, while the bacteria numbers started to decrease very slowly after reaching a maximum. A very late gradual secondary rise in the oxygen uptake was noticed generating a long plateau in the oxygen uptake curve. The glucose removal in the BOD bottle was followed by the anthrone test after concentrating the samples. The substrate was eliminated from the system by the time the first stage of oxygen uptake was exerted and the bacterial population reached its peak.

In the experiment shown in Fig. 12 an attempt was made to remove or mask the plateau by starting with a higher number of protozoa in the seed inoculum than is usually present in sewage seed. The seed for this experiment was obtained as described in the "protozoa-rich seed" preparation procedure in Materials and Methods (Chapter IV). Ammonia nitrogen was used for this seed preparation, and the cells were harvested at 36 hours instead of 48 hours, as previously described in the procedure. Before use in the BOD experiment the cells were washed twice in 0.05 M phosphate buffer pH 7. Partial success was attained in removing the pause (or plateau) from the oxygen uptake curve. The protozoa developed quite rapidly with respect to the bacterial population growth in this system as compared to the previous experi-

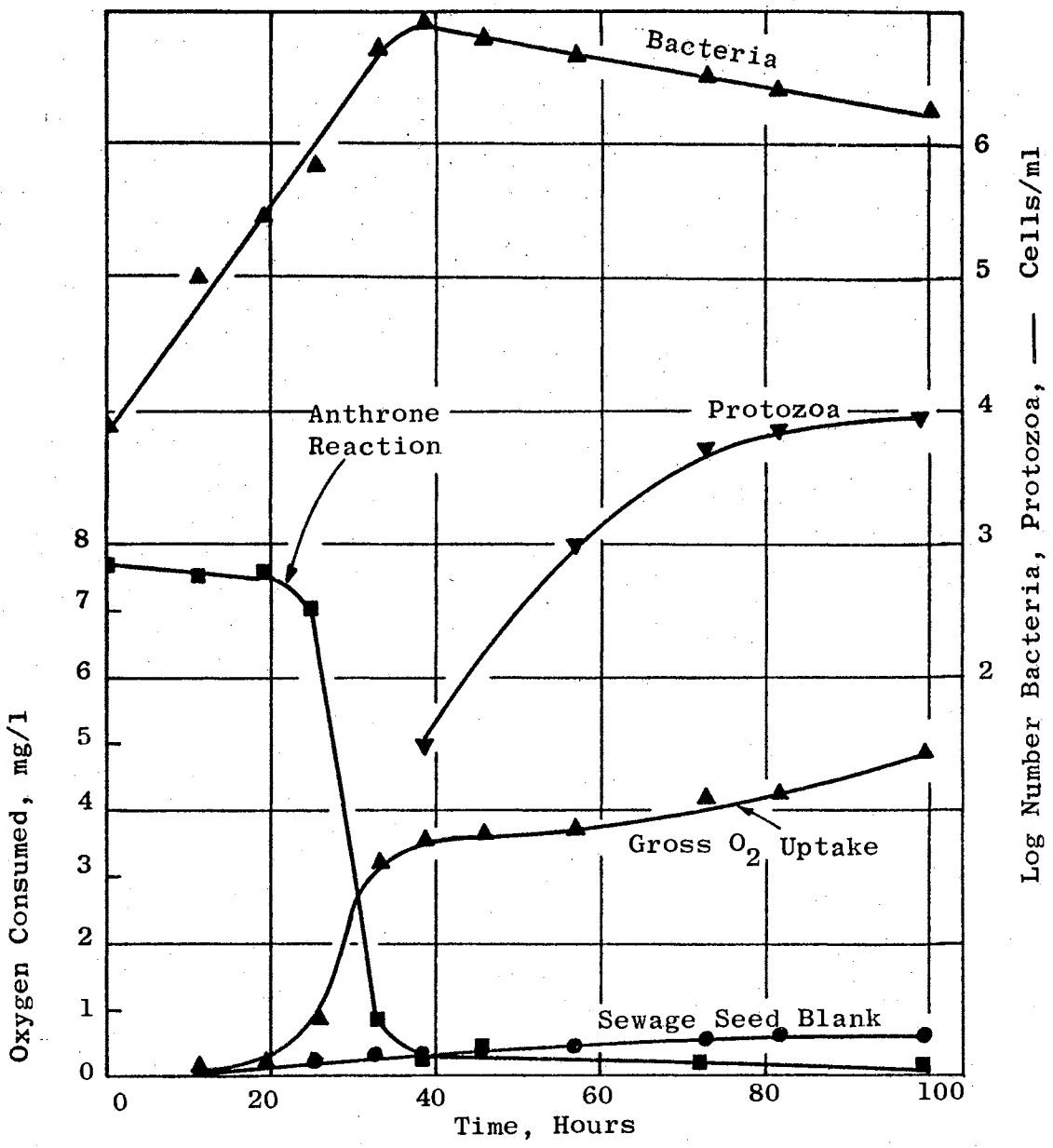


Fig. 11 - BOD EXERTION, SUBSTRATE REMOVAL, BACTERIA AND PROTOZOA COUNTS IN THE BOD BOTTLE USING SEWAGE SEED - GLUCOSE CONC. 7.7 mg/l.

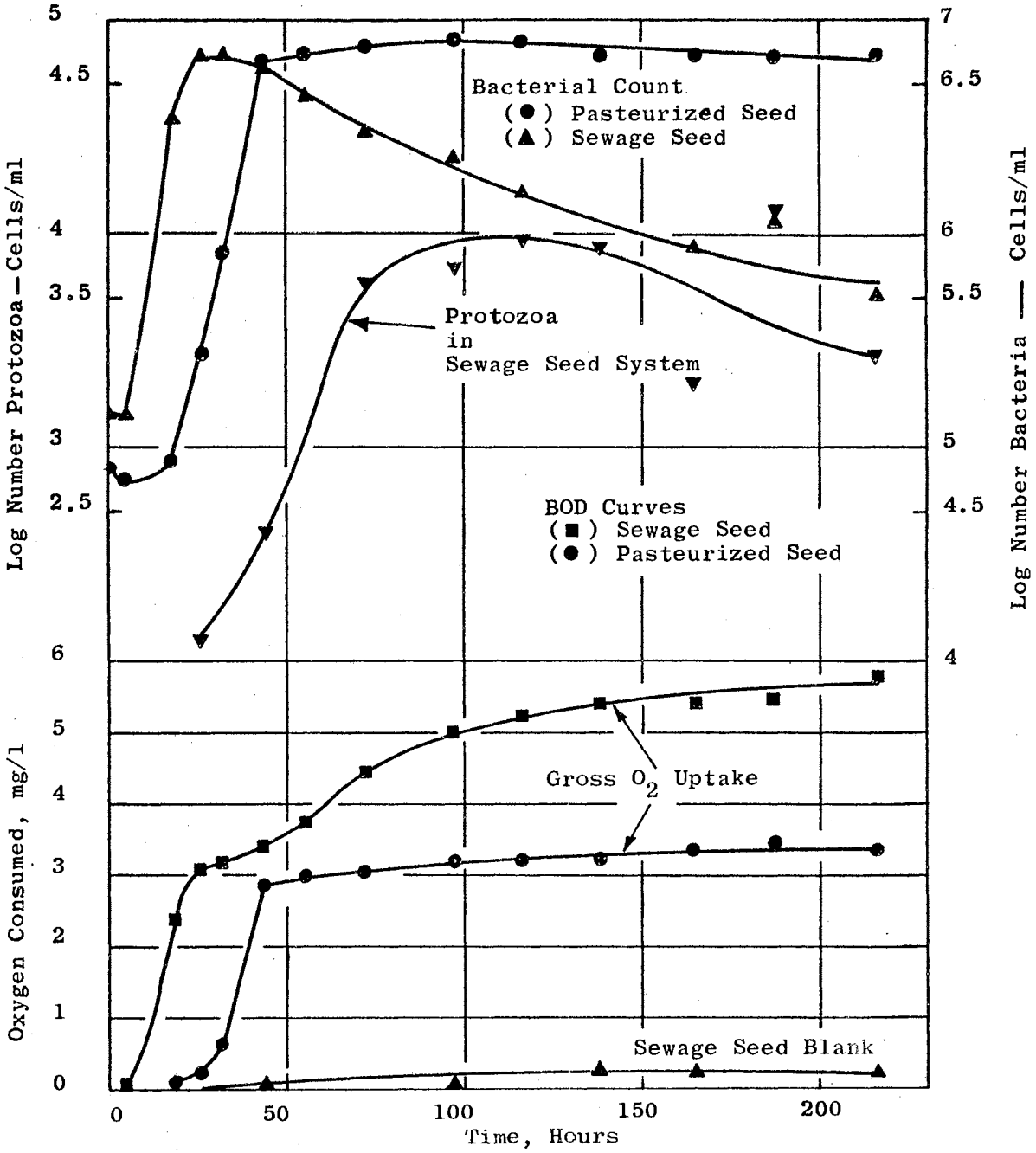


Fig. 12 - BOD EXERTION, BACTERIA AND PROTOZOA COUNTS IN THE BOD BOTTLE, USING 8 mg/l GLUCOSE.

ment (Fig. 11). A rapid decline in the viable bacterial population was observed. On the other hand, the pasteurized seed system showed no second stage of oxygen uptake and the bacterial count never dropped after reaching a limiting number.

From the results presented above on the oxygen uptake and the bacterial and protozoan population dynamics in high and low energy systems it would seem that the relative pattern of changes in the bacteria and protozoa population determine the shape of the oxygen uptake expression with respect to the occurrence and the magnitude of the plateau. Whereas the presence of protozoa is essential for the exertion of the second stage of oxygen utilization, the appearance and the severity of a plateau between the two stages of oxygen uptake depends solely upon the relative times of growth of the bacteria and protozoa in the system. In other words, plateau length depends upon the time lag between the peaks in bacteria and protozoa populations. The smaller the lag, the smaller the plateau; the longer the lag, the longer the plateau. In general, the second stage of oxygen uptake is accompanied by a rapid increase in the protozoa population, and the initiation of the second stage of oxygen uptake would in these systems seem to be governed by the presence of an adequate number of active protozoa. The relationship between the different phases of oxygen uptake and the viable bacteria counts for the results

reported here are in agreement with the previous findings of the author in the dilute systems (the BOD bottle)(4)(5).

D. Influence of the Protozoa-Bacteria Ratio in the Inoculum on Oxygen Uptake

A seed dilution method was employed to prepare seed material which was successively smaller in numbers of protozoa. In order to maintain nearly the same bacterial population in the final seed material, a pasteurized seed was used as a base to which different dilutions of the protozoa containing seed were added. The procedure for the preparation of seeding material is described in Materials and Methods (Chapter IV). The letter "S" is used for the seed preparation derived from the sewage seed, and the suffix number stands for the dilution factor for protozoa as compared to the parent sewage seed. For example, "S_5" means a seed with five times less protozoa than in the sewage seed.

The results for the effect of different protozoa-bacteria ratios in the seed on oxygen uptake in high energy systems are shown in Fig. 13. Five - (S_5) and ten - (S_{10}) fold dilution of the initial protozoan population in the sewage seeds caused an extension of the plateau in the oxygen uptake curve, while dilutions of one hundred (S_{100}) and two hundred (S_{200}) fold completely eliminated the second stage of oxygen utilization. The results

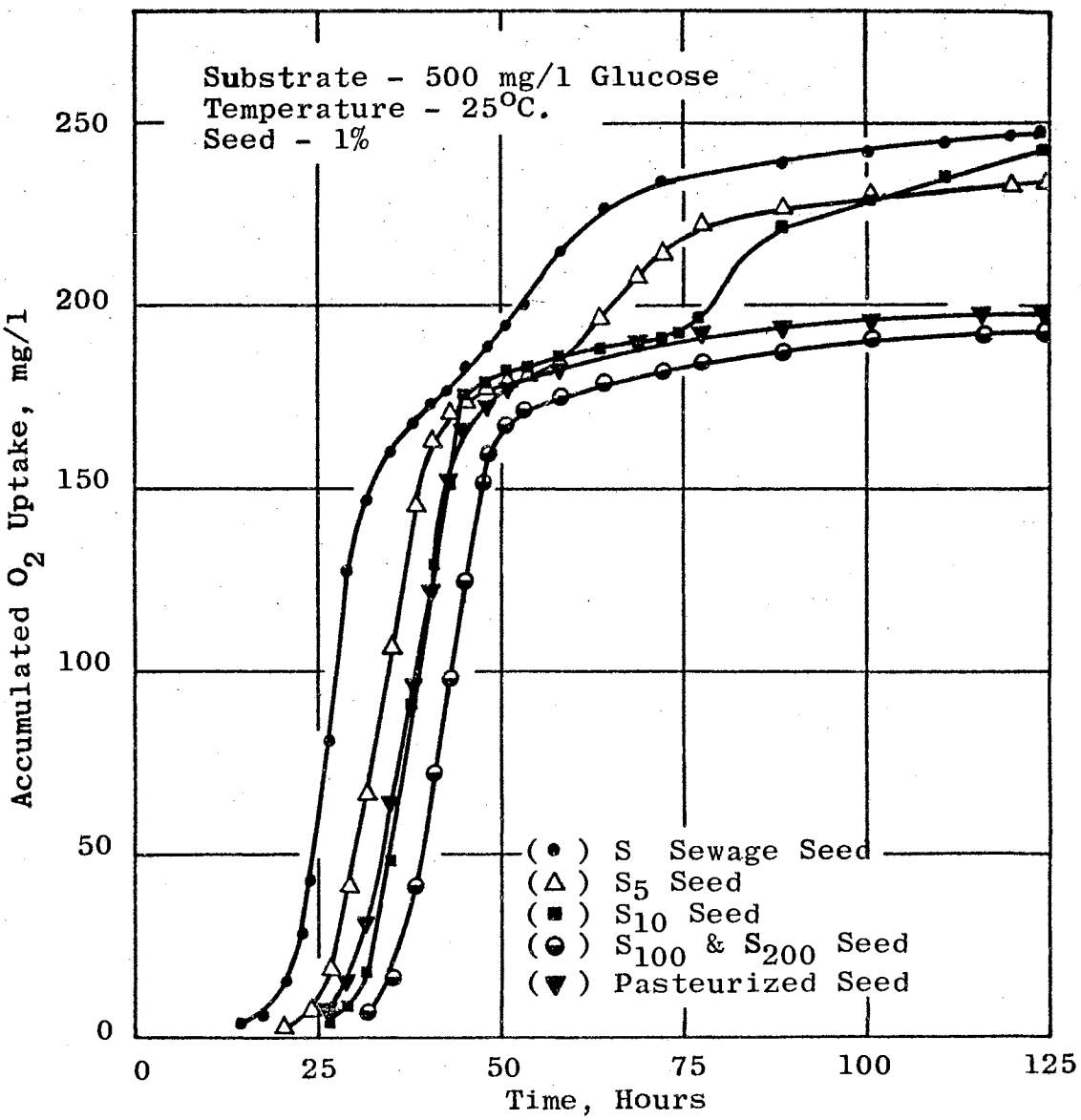


Fig. 13 - WARBURG STUDIES USING DIFFERENT PROTOZOA-BACTERIA RATIOS IN SEED PREPARATIONS.

obtained with the S_{100} and S_{200} seeds were comparable to the results obtained with the pasteurized seed.

Similar elongation of the pause was evidenced using S_{10} seed material during BOD exertion in low energy systems (the BOD bottle) as compared to the sewage seed system (see Fig. 14). The oxygen utilization during the second stage by the system containing a lower initial amount of protozoa was less than in the sewage seed system. Also, the bacterial count decreased less rapidly and by a lesser amount for the diluted seed as compared to the sewage seed system (S) which showed a greater and more rapid loss of the viable bacterial population. The pasteurized seed system showed no second oxygen uptake stage and no dieoff of the bacterial cells was noticed after reaching a maximum count.

E. Interaction of Bacteria and Protozoa - Mixed Bacterial Systems

Another pertinent Warburg study was designed to elucidate the effect of the presence of protozoa in the bacterial system on the expression of oxygen uptake. The experimental design consisted of studying the effect of addition of protozoa to a mixed bacterial culture during the course of oxygen uptake.

A series of Warburg flasks containing 500 mg/l glucose synthetic waste was inoculated with a pasteurized glucose-acclimated seed, and the oxygen uptake was followed. At the

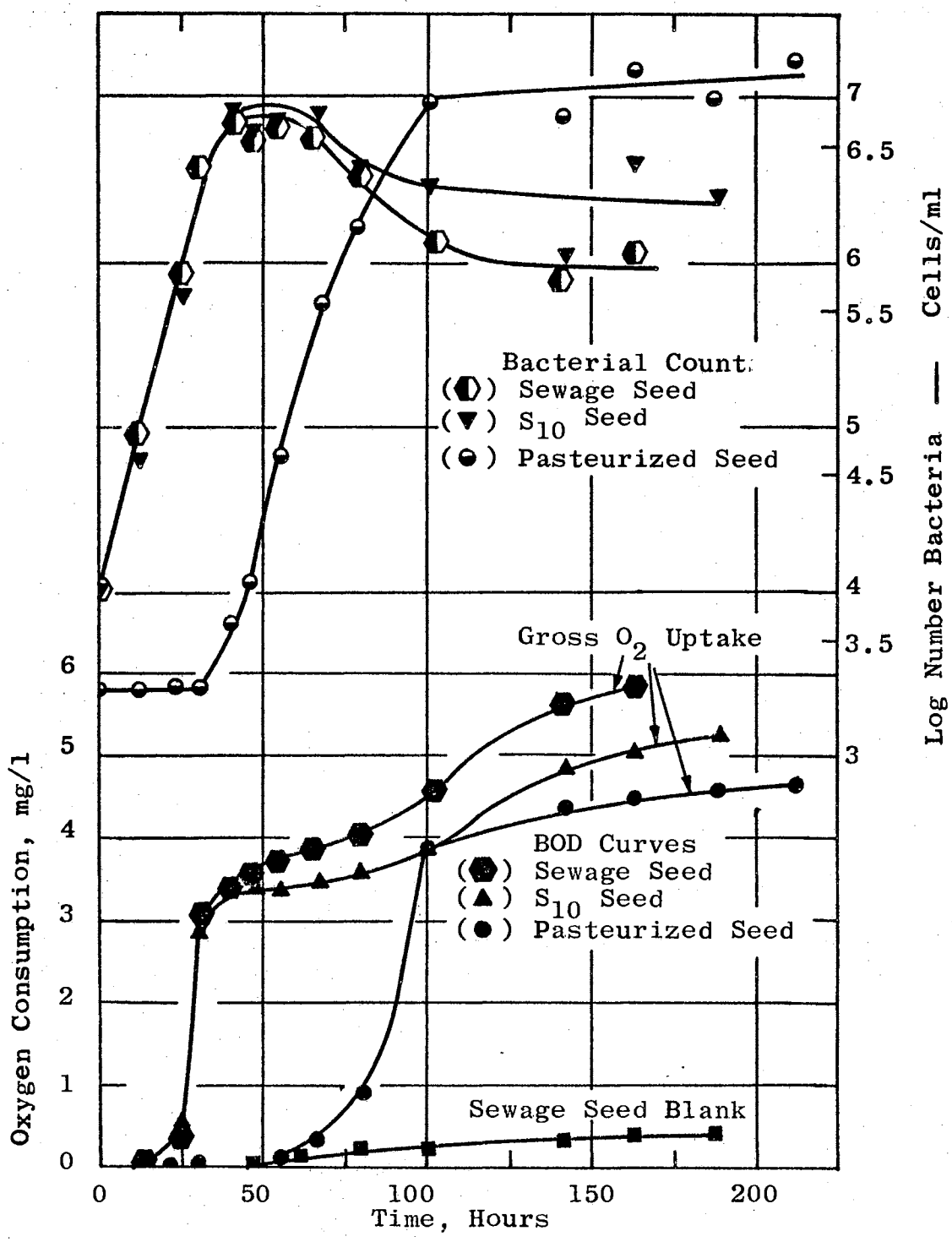


Fig. 14 - BOD EXERTION AND BACTERIA COUNT IN THE BOD BOTTLE, USING DIFFERENT PROTOZOA-BACTERIA RATIOS IN SEED PREPARATION. GLUCOSE CONC. 9 mg/l.

end of 24 hours (see arrow Fig. 15) when the rapid oxygen uptake in the system had ceased, a number of flasks were inoculated with one ml of a "protozoa-rich inoculum". Flask A was followed as a control. It is seen in Fig. 15 that a secondary oxygen demand was observed in the protozoa-inoculated system (Flask B). Flask B consumed 97 mg/l of oxygen in excess of the control Flask A, indicating that 29.5 per cent of the total oxygen consumption in Flask B was due to the addition of protozoa. The protozoan count in Flask B increased from 1.3×10^4 cells/ml at the time of inoculation to a maximum of 1.4×10^6 cells/ml. The maximum recorded protozoan population was observed to occur when nearly 50 per cent of the total secondary oxygen uptake had been exerted. An insignificant amount of oxygen was consumed by the protozoan inoculum control.

A parallel study was conducted in dilute or low energy systems, i.e., the BOD bottle. The pasteurized seed control bottles using 9 mg/l glucose as substrate were inoculated with one ml of "protozoa-rich inoculum" at the time indicated by an arrow in Fig. 16. Two different inoculations were made. System B was inoculated with one ml of 1/10 dilution of the protozoan inoculum used for system A. BOD exertion, and bacterial and protozoan counts are also shown in Fig. 16. While both systems A and B showed a second stage of oxygen uptake, the second stage in system B lagged that in system A. In like manner the protozoan development

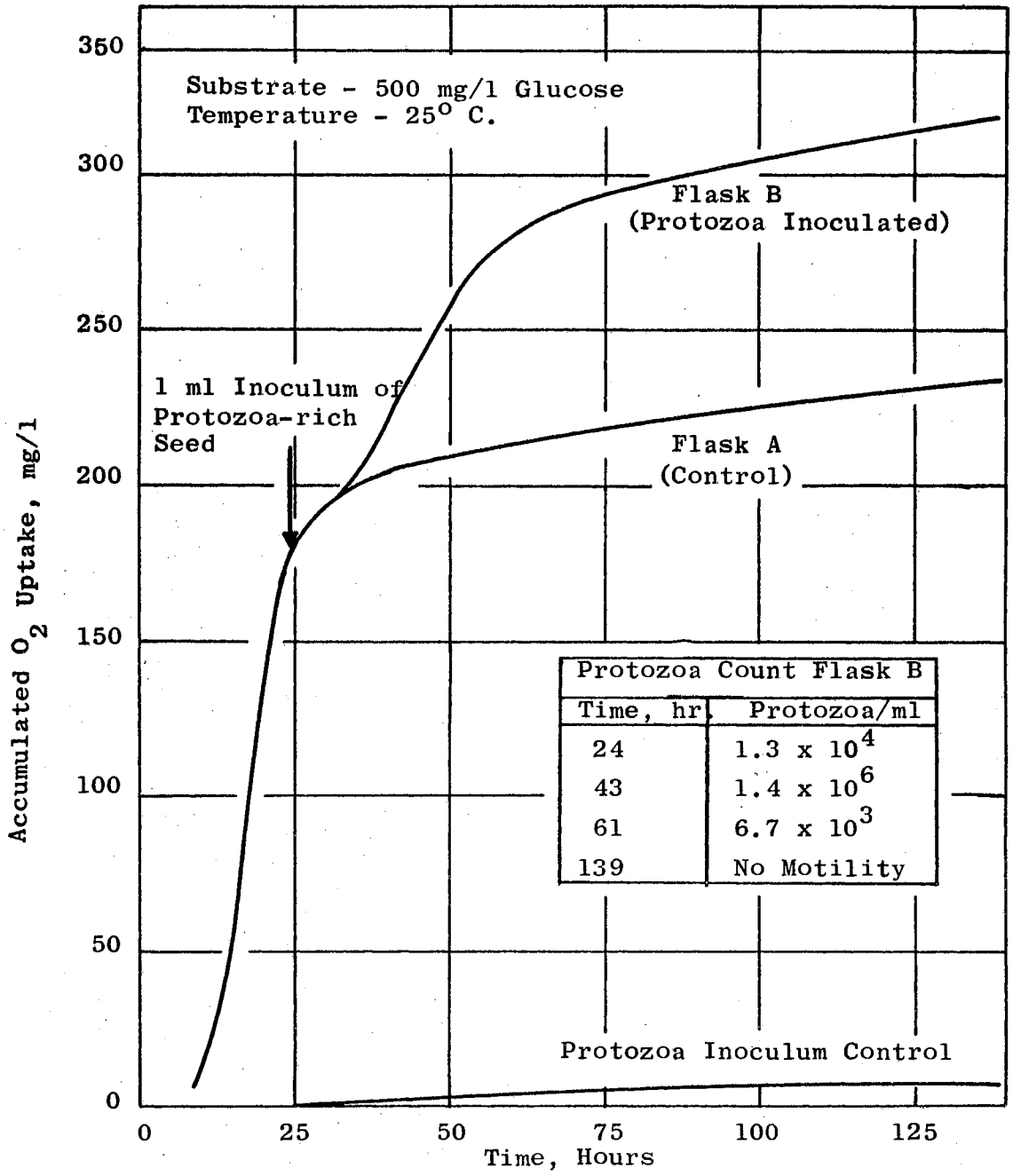


Fig. 15 - WARBURG STUDY, USING SMALL INOCULUM OF ACCLIMATED PASTEURIZED SEED.

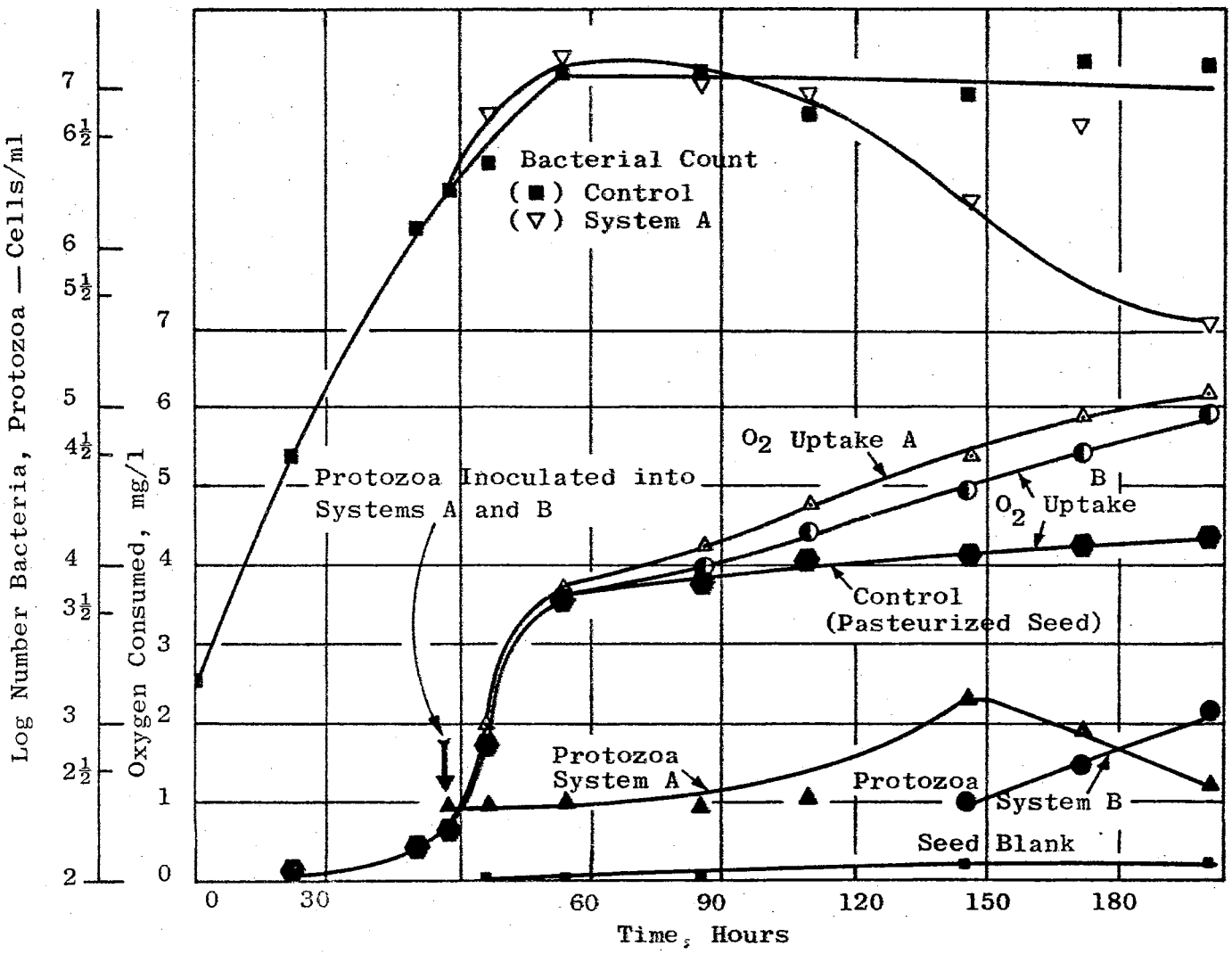


Fig. 16 - BOD EXERTION, BACTERIA AND PROTOZOA COUNTS IN THE BOD BOTTLE, USING 8 mg/l GLUCOSE. BOD VALUES ARE GROSS VALUES.

toward maximum population in system B also lagged the protozoan growth in system A. It is interesting to note that the maximum protozoan population reached in either system was much less (in the order of 1000) as compared to experiments (Figures 8, 11 and 12) earlier reported (in the order of 10,000). The pasteurized control showed no second stage of oxygen uptake. The decline in bacterial count in system A corresponded to the secondary rise in oxygen uptake. A decrease in viable bacterial population after reaching a maximum count was not registered in the control. The protozoan inoculum control used only a small amount of oxygen.

Microscopic examination of protozoa revealed only ciliates of Enchelys type. These protozoa are large in size (25-50μ). This might account for the somewhat low protozoan count observed.

F. Effect of Predators on Biochemical Response

Long term aeration studies were carried out on the Warburg apparatus using a sewage seed and a pasteurized sewage seed for the purpose of comparing the system's biological response with regard to substrate removal, solids production, intra-cellular protein content and oxygen uptake in the presence and absence of predators. The seed material employed for the two experiments reported in Figures 17 and 18 were from different sewage samples; the experiments were run approximately six months apart.

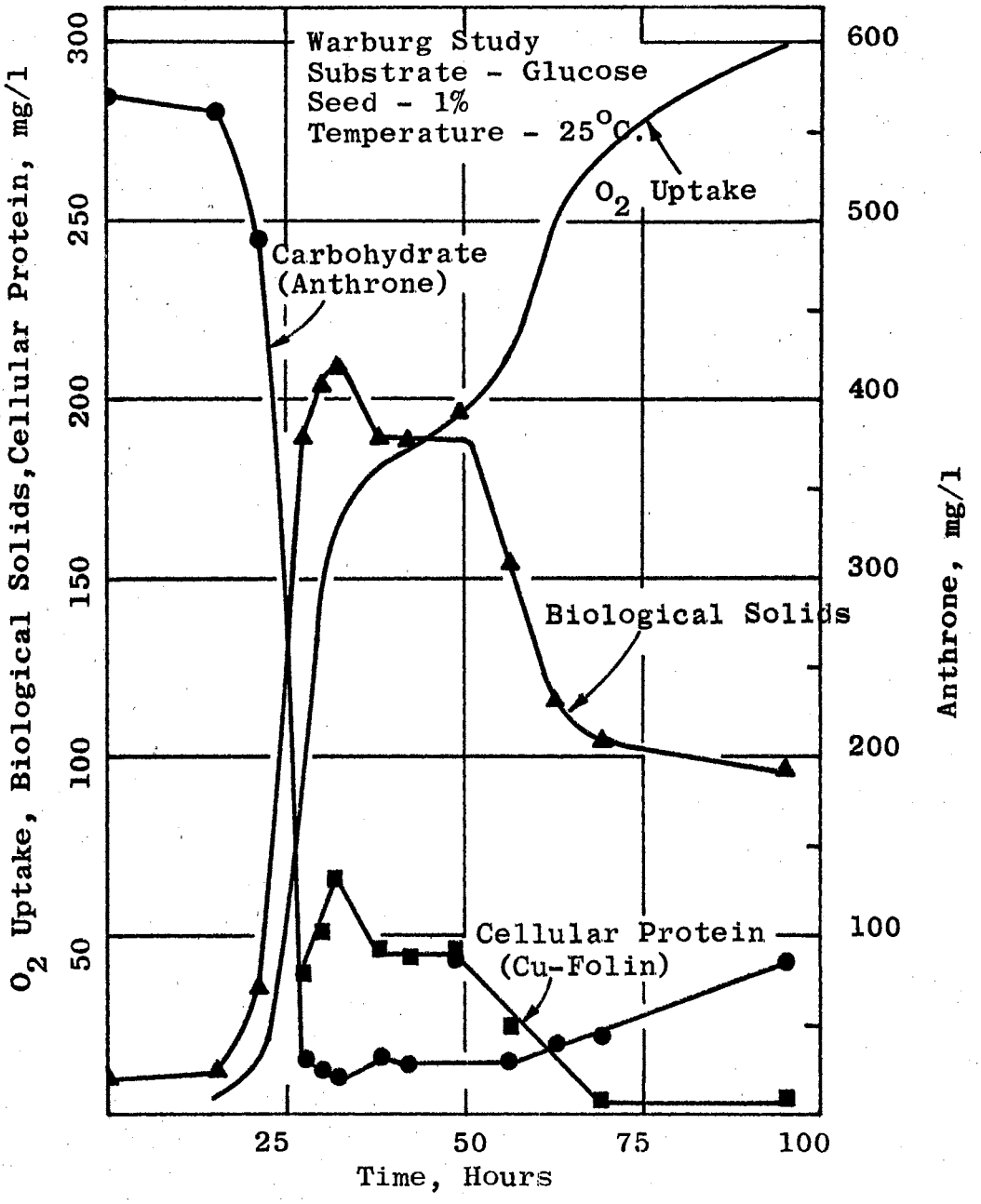


Fig. 17 - LONG TERM AERATION STUDY, USING SEWAGE SEED.

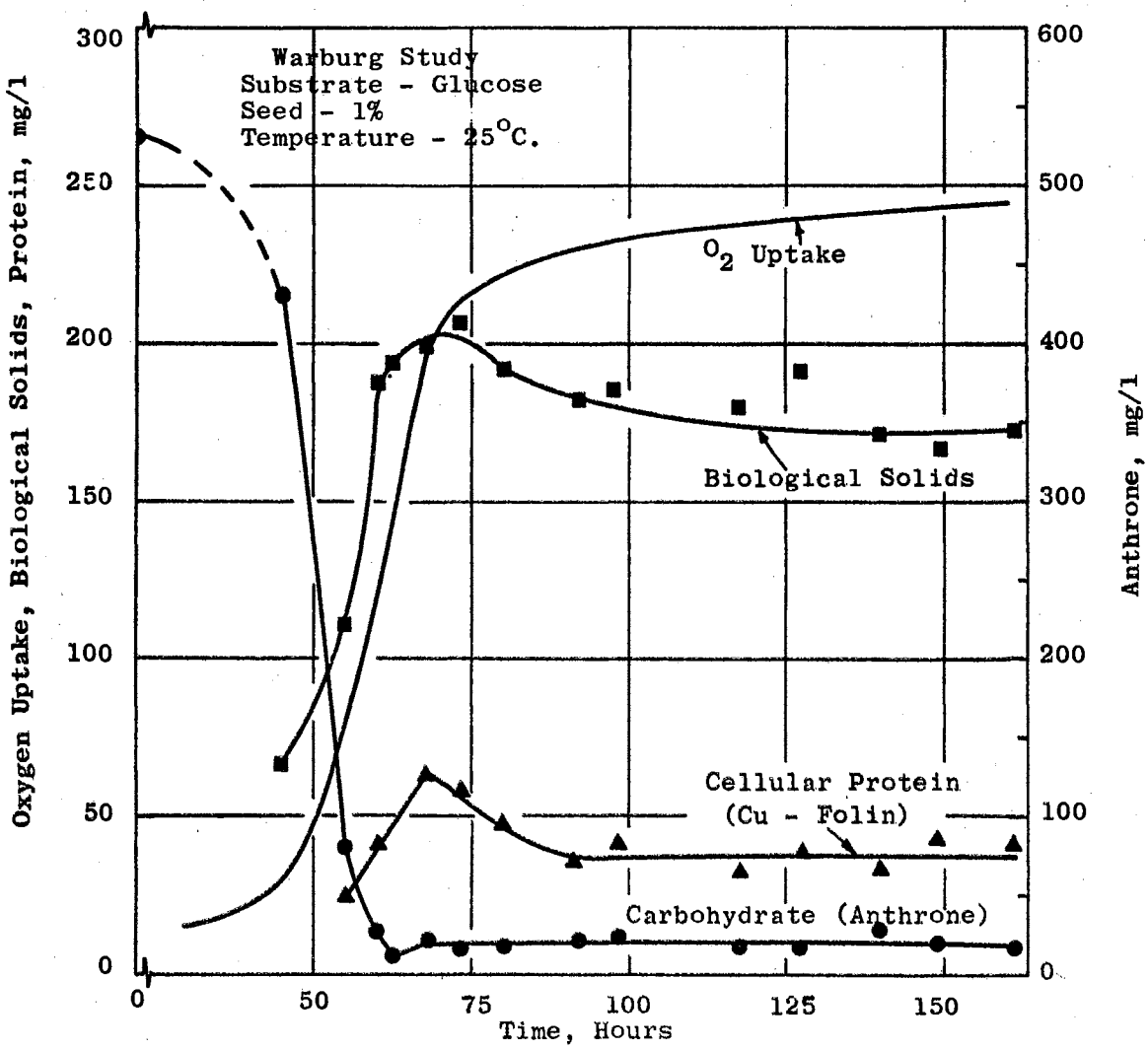


Fig. 18 - LONG TERM AERATION STUDY, USING PASTEURIZED SEWAGE SEED.

Fig. 17 shows the pattern of substrate removal, biological solids production and cellular protein during oxygen uptake using one per cent sewage seed. It is seen that glucose, as measured by the anthrone reaction, was exhausted from the system slightly before occurrence of the peak in biological solids. A considerable rise in anthrone reactive material was noticed after 56 hours, during the second stage of oxygen uptake. Maxima in solids concentration and cellular protein content occurred simultaneously, before the end of the first stage of oxygen uptake, and then each decreased by 20 mg/l before the beginning of the second stage of oxygen utilization. No apparent change in solids or cellular protein content was observed during the occurrence of the plateau in the oxygen uptake. However, both biological solids and cellular protein decreased rapidly during the second stage of oxygen utilization. At the termination of the experiment 96 mg/l of solids and 4 mg/l of protein were recorded in the system.

Results reported in Fig. 18 were obtained using one per cent of the pasteurized sewage seed. Maximum glucose removal measured by the anthrone determination occurred before the solids peak, as in the last experiment using the sewage seed (Fig. 17). However, in this case no rise in anthrone-reactive material was observed in the later part of the experiment, and the oxygen demand was exerted in a single stage. Both biological solids and cellular protein,

after reaching a maximum value, simultaneously decreased 20 mg/l. This loss continued until 90 hours on the time scale. Beyond this time solids decreased very slightly, and there was no discernible loss in cellular protein content. At the end of the experiment 176 mg/l of solids, 40 mg/l of which were protein, were noted in the system.

G. Interaction of Bacteria and Protozoa - Pure Bacterial Culture

Finally, Warburg studies were made in which protozoa were inoculated into suspensions of a pure culture bacterial system (devoid of substrate) in order to determine the contribution of protozoa to the so-called endogenous respiration of the mixed population system. A washed suspension of a pure culture of Serratia marcescens was used in these investigations.

Fig. 19 shows oxygen uptake, and bacterial and protozoan counts for a protozoa-inoculated and a control system. The Serratia marcescens control consumed less than 15 mg/l of oxygen during the experiment, and throughout this period no loss in the viable bacterial count was observed. However, the protozoa-inoculated flask consumed about 110 mg/l of oxygen and exhibited a very large decline in the viable bacterial count, i.e., from a maximum of 1.3×10^9 cells/ml to a minimum of 7.25×10^6 cells/ml. The expression of oxygen uptake followed an autocatalytic type curve. The protozoan

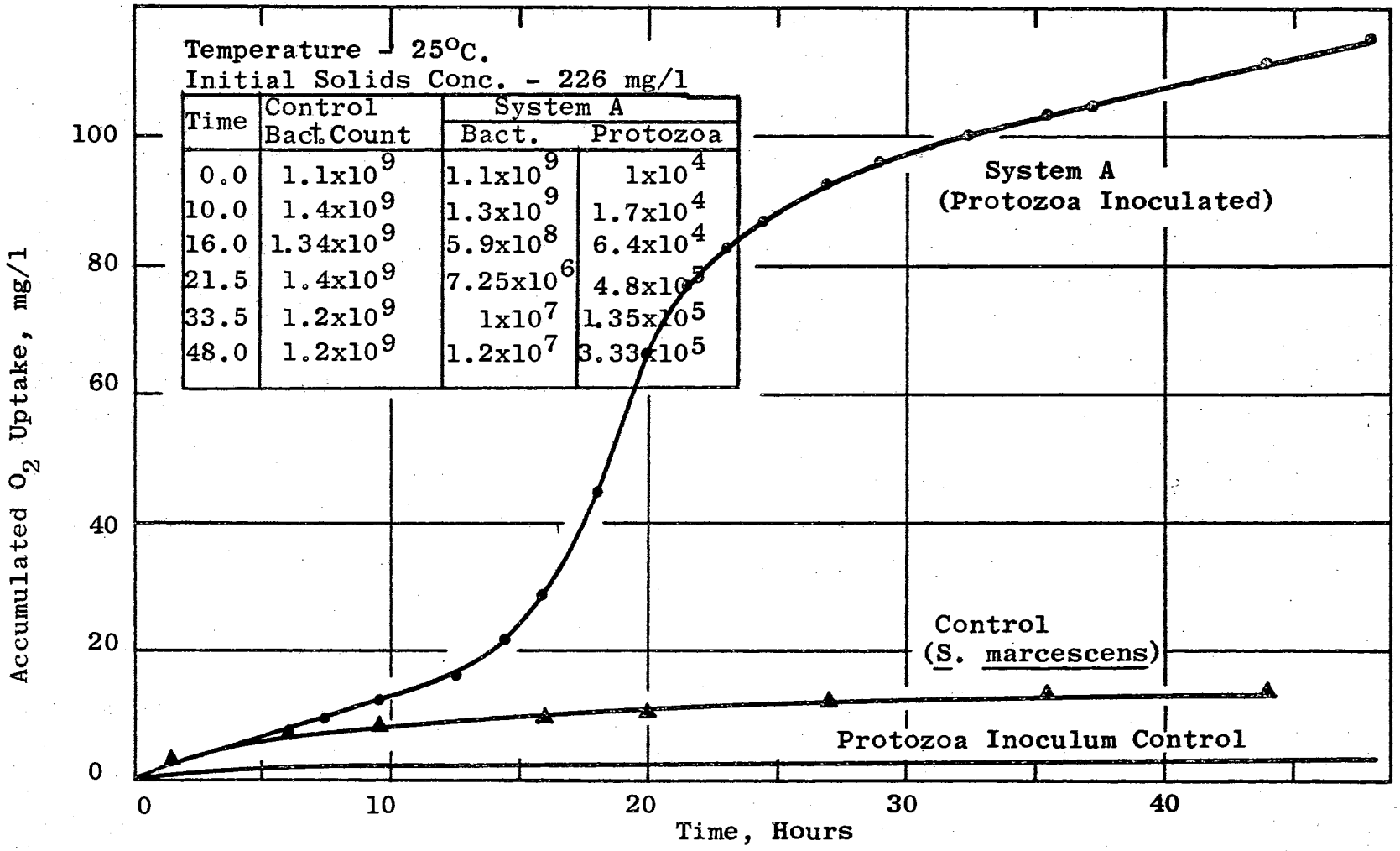


Fig. 19 - WARBURG STUDIES ON THE INTERACTION OF A PURE BACTERIAL CULTURE AND PROTOZOA. BACTERIA AND PROTOZOA COUNTS AT DIFFERENT TIMES ARE GIVEN IN TABULAR FORM ON THE FIGURE.

count increased from 1 x 10^4 cells/ml at the beginning of the experiment to a recorded maximum of 4.8 x 10^5 cells/ml. A bacterial carryover count of 2.9 x 10^6 was registered due to the protozoan inoculum. The bacterial count other than Serratia marcescens, which could be recognized due to the red colonies characteristic of S. marcescens, was found to be 1.1 x 10^7 cells/ml at the end of 48 hours. The oxygen consumption by the protozoan control was negligible.

A quantitative approach to assessment of the amount of oxygen utilization in the second stage due to metabolism of bacteria by the protozoa was made in the experiment shown in Fig. 20. A series of Warburg flasks containing different initial solids concentrations of a pure culture cell suspension were inoculated with a "protozoa-rich inoculum". The oxygen uptake results for the various systems and the controls are shown in Fig. 20. The amount of oxygen consumption in the protozoa-inoculated flasks containing 249 mg/l and 592 mg/l of initial solids was found to be proportional to the initial solids concentration. But the flask containing 1185 mg/l initial solids concentration showed a sudden cessation of oxygen utilization, for some reason, at a somewhat lower value of oxygen consumption than could be expected in accordance with the initial cells concentration present. A visual examination of the contents of the flasks at the end of the experiment revealed a very high degree of clarification in flasks containing 249 mg/l

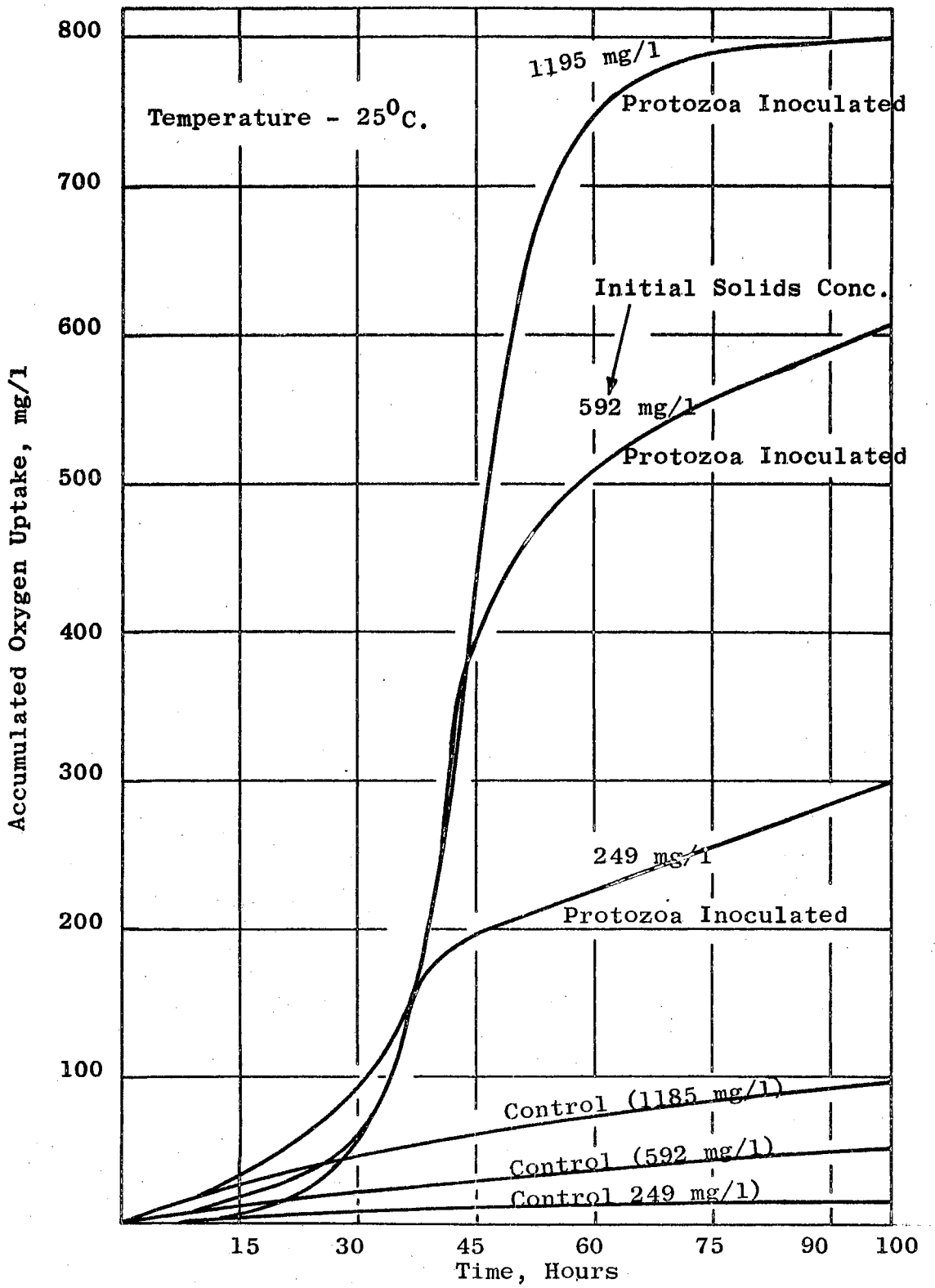


Fig. 20 - WARBURG STUDIES ON THE INTERACTION OF A PURE BACTERIAL CULTURE AND PROTOZOA USING DIFFERENT INITIAL SOLIDS CONC. OF BACTERIUM S. marcescens.

and 592 mg/l of solids as compared to the flask with 1185 mg/l of initial solids.

The results reported in Figures 19 and 20 suggest that the so-called endogenous respiration, in the presence of predators (protozoa) is due mainly to the metabolism of protozoa feeding on bacteria, and that the contribution of bacteria to the total oxygen uptake in the second stage is very small. These results also suggest that the so-called endogenous respiration in such systems is proportional to the bacterial cells present simply because the bacterial cells serve as substrate to the predators.

Investigations Related to Theory 3

Results reported in this portion pertain to investigations made to assess the role of Theory 3 in the causation of the plateau.

Theory 3 states that the second phase of oxygen utilization is due to the metabolism of intracellular substance, and the appearance of the plateau in O_2 uptake is due to the time required for the formation of the enzyme system essential for the metabolism of the cellular material.

Table III shows the effect of the addition of chloramphenicol in concentrations of 10 mg/l and 50 mg/l on bacterial growth and cellular protein synthesis in an actively metabolizing activated sludge. Growth (as replication) in the system was determined as an increase in the

TABLE III

EFFECT OF CHLORAMPHENICOL (10 and 50 mg/l) ON GROWTH AND PROTEIN SYNTHESIS IN BATCH ACTIVATED SLUDGE UNITS

Time	D. W. Solids, mg/l			Viable Cell Count, mg/l			Protein, mg/l		
Hours	Control	10 mg/l	50 mg/l	Control	10 mg/l	50 mg/l	Control	10 mg/l	50 mg/l
0.0	60			8.6×10^7			26		
3.0	170			1.4×10^8			75		
5.0	304			3.7×10^8			114		
6.0	-	300	308	-	3.1×10^8	3.0×10^8	-	120	117
6.5	372	-	-	4.2×10^8	-	-	136	-	-
7.0	-	313	300	-	3.5×10^8	3.5×10^8	-	144	120
8.0	427	340	-	7.2×10^8	3.6×10^8	-	187	128	-
9.0	-	353	307	-	2.1×10^8	1.6×10^8	-	128	120
10.0	460	-	340	8.1×10^8	-	1.6×10^8	214	-	107
11.0	-	390	-	-	2.3×10^8	-	-	133	-

Chloramphenicol was added at the end of five hours

viable bacterial count. The changes in dry weight of cells were also recorded. Cellular protein content was determined by the Copper-Folin method. After treatment with chloramphenicol the viable bacterial count in both systems to which the inhibitor had been added showed no increase. While one doubling of the population was noted in the control from the time the chloramphenicol was added until the end of the experiment, the viable bacterial population in the chloramphenicol treated systems showed a small drop during this time. For all practical purposes protein synthesis was completely inhibited in both chloramphenicol treated systems as compared to the control; however, the inhibition was more complete at the 50 mg/l concentration. The increase in dry weight of cells after treatment with chloramphenicol was curtailed.

A separate study was conducted to determine the effect of various concentrations of chloramphenicol on the growth of protozoa in an activated sludge system similar to the one used in the previous experiment. The results are shown in Table IV. The figures in the columns show the protozoan count in the system prior to and after treatment with various concentrations of chloramphenicol (50 to 500 mg/l) at nine hours after the beginning of the experiment. An increase of approximately 10^6 protozoa cells/ml was recorded after treatment with chloramphenicol in each system.

TABLE IV

EFFECT OF CHLORAMPHENICOL (50 - 500 mg/1) ON PROTOZOAN GROWTH IN BATCH ACTIVATED SLUDGE UNITS

Time Hours	Protozoa Cells/ml						
	0	5	9	15	25	31	39
Control	1.33×10^3	2.67×10^3	5.1×10^3				
50 mg/1+				6.3×10^3	4.25×10^4	1.97×10^5	1.13×10^6
100 mg/1+				7.5×10^3	2.95×10^4	2.05×10^5	8.4×10^5
200 mg/1+				8.4×10^3	2.5×10^5	2.05×10^6	2.9×10^6
500 mg/1+				8.7×10^3	3.75×10^4	1.8×10^5	1.1×10^6

Chloramphenicol was added at the end of nine hours

\+ Chloramphenicol concentration in the system

It was concluded from the above two investigations that chloramphenicol concentrations of 10 and 50 mg/l were effective in inhibiting the bacterial replication and protein synthesis in activated sludge units; however, chloramphenicol did not interfere with the growth of protozoa up to a concentration of 500 mg/l.

Figures 21 and 22 show accumulated oxygen uptake for a glucose synthetic waste inoculated with acclimated and sewage seed respectively. Chloramphenicol in concentrations of 10 and 50 mg/l was tipped into the Warburg flasks well before the break in the oxygen uptake (i.e., before the plateau). In the experiment reported in Fig. 22 the biological solids, COD and carbohydrate in the filtrate were determined at the point of addition of chloramphenicol, and are listed on the figure. It is seen that tipping of the chloramphenicol into the flasks under these circumstances did not materially affect the second stage of oxygen uptake with either acclimated or sewage seeds, as compared to the control systems.

PHASE II - Investigation into the Causation of the Plateau in Pure Culture Systems

Oxygen Uptake Studies on Pure Cultures

Figures 23, 24 and 25 show accumulated oxygen uptake by pure cultures (obtained from the department of microbiology

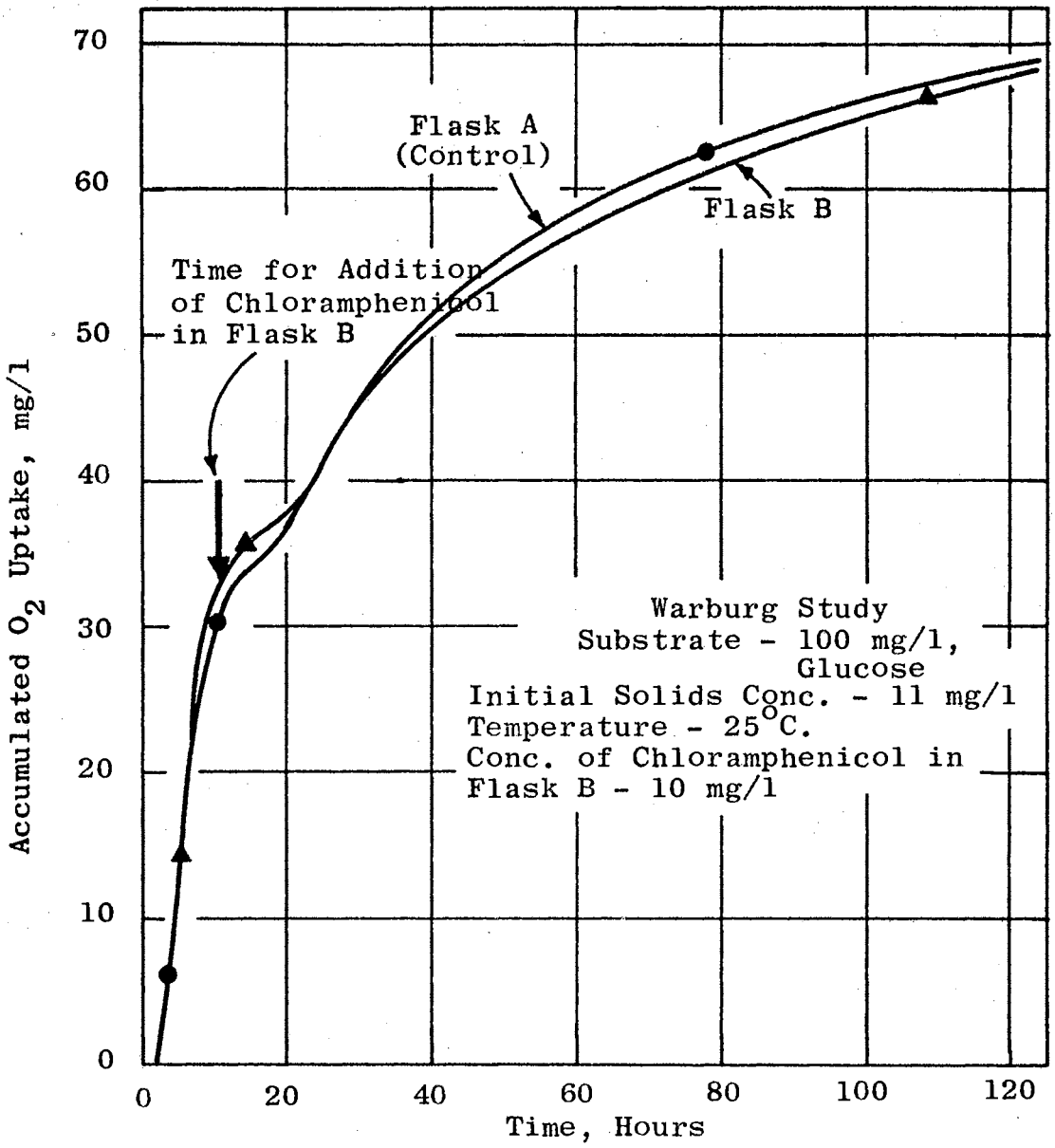


Fig. 21 - EFFECT OF THE PROTEIN SYNTHESIS INHIBITOR CHLORAMPHENICOL ON O_2 UPTAKE USING ACCLIMATED SEED.

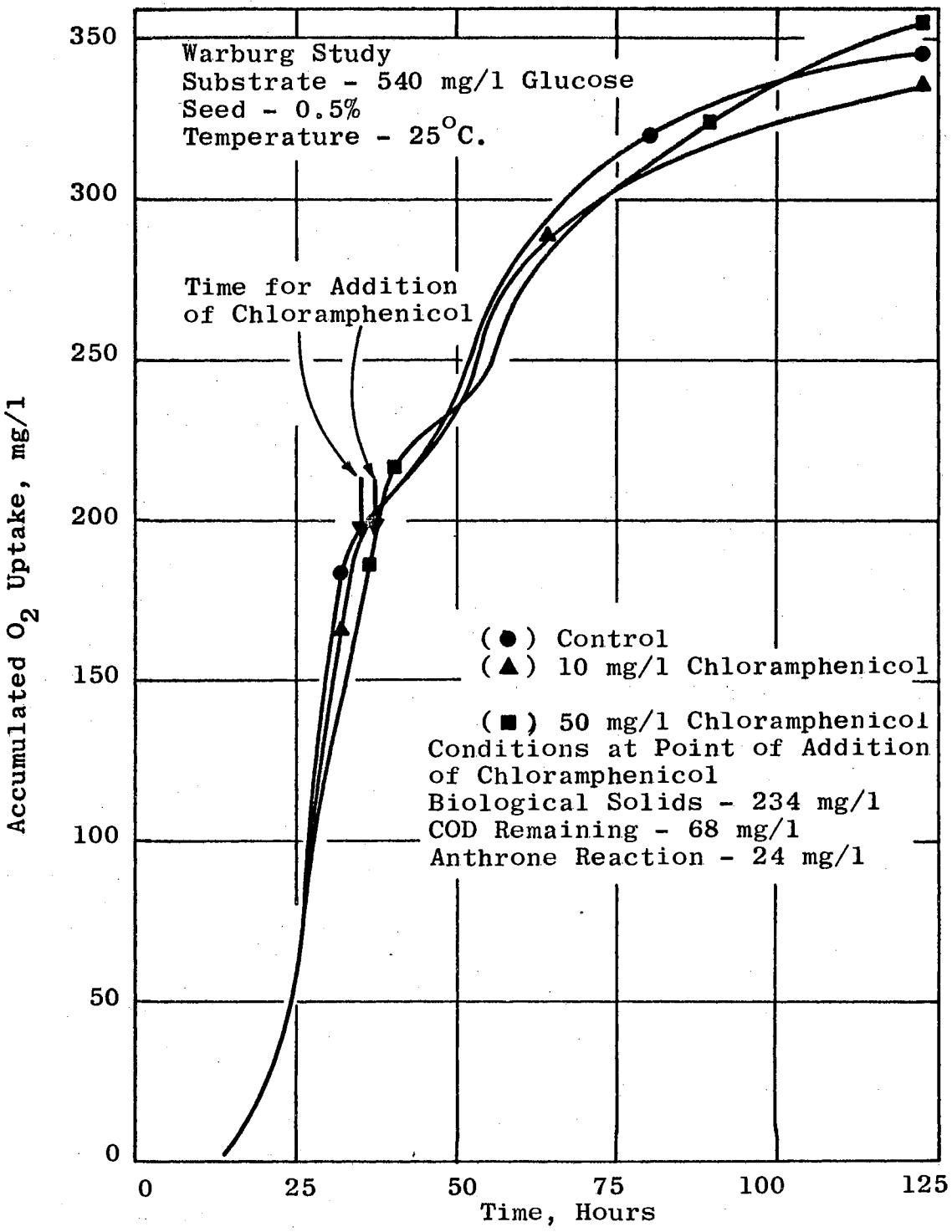


Fig. 22 - EFFECT OF THE PROTEIN SYNTHESIS INHIBITOR CHLORAMPHENICOL ON O_2 UPTAKE USING SEWAGE SEED.

at Oklahoma State University) using glucose as the sole source of carbon. It is seen that the oxygen uptake pattern did not show two stages of oxygen uptake separated by a plateau; however, the kinetics of the oxygen uptake observed consisted of a phase of rapid uptake followed by a phase of slow uptake. In all, nine strains of Escherichia coli and eight strains of Pseudomonas species were examined, but due to the general similarity in the shape of the oxygen uptake curve not all the results are plotted. A total of 21 pure cultures were examined.

Next, pure cultures of bacteria were isolated from heterogeneous populations used in the previous Warburg experiments (Phase I), which showed a typical two-stage oxygen utilization and a plateau. Fig. 26 shows results from three such pure cultures. Since these cultures were obtained from reaction mixture employing nitrate as nitrogen source, the oxygen uptake measurements were also made in this case using $NaNO_3$ in the medium. The oxygen uptake on a combination of cultures No. 4, 5, and 6 mixed in equal proportions is also reported in Fig. 26. The results for all of these systems again showed two phases of oxygen uptake; a rapid phase followed by a slow phase. Two more pure cultures were isolated from another Warburg experiment. The oxygen uptake curves were similar in shape to those expressed in Fig. 26. A total of five pure cultures was examined in this set of experiments.

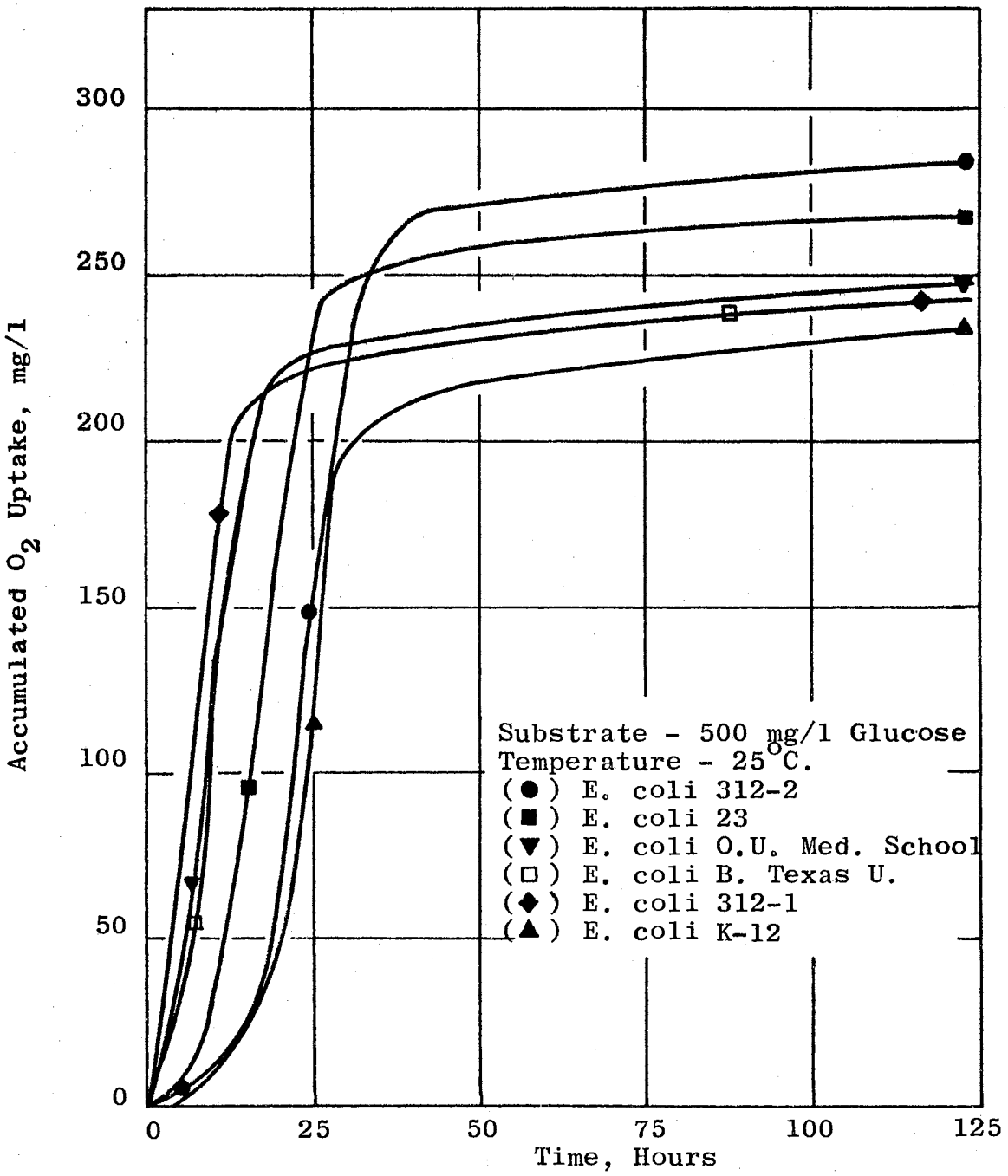


Fig. 23 - WARBURG STUDIES ON VARIOUS STRAINS OF Escherichia coli, USING ACCLIMATED SMALL INITIAL INOCULUM.

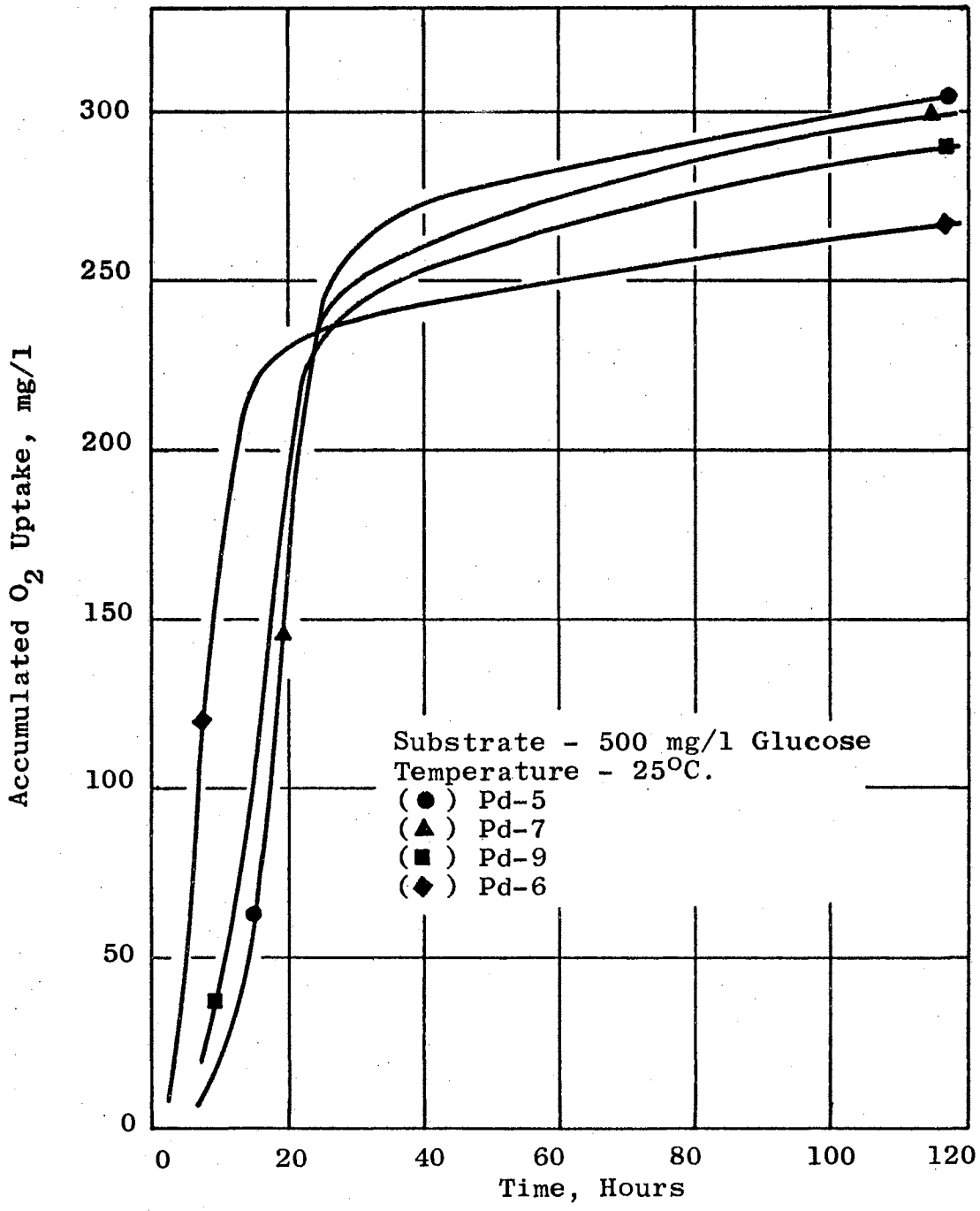


Fig. 24 - WARBURG STUDIES ON VARIOUS STRAINS OF Pseudomonas sp., USING ACCLIMATED SMALL INITIAL INOCULUM.

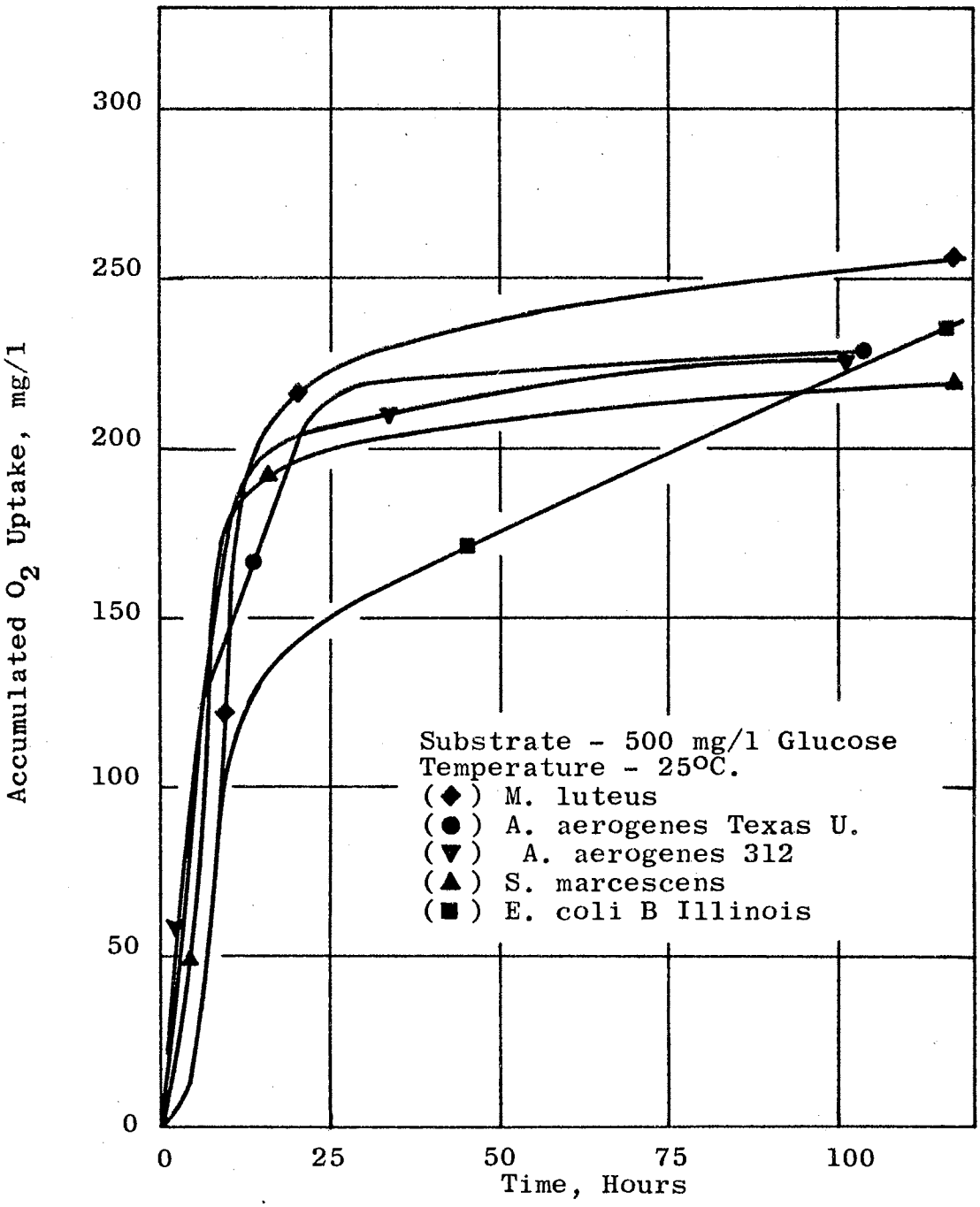


Fig. 25 - WARBURG STUDIES ON VARIOUS PURE CULTURES, USING ACCLIMATED SMALL INITIAL INOCULUM.

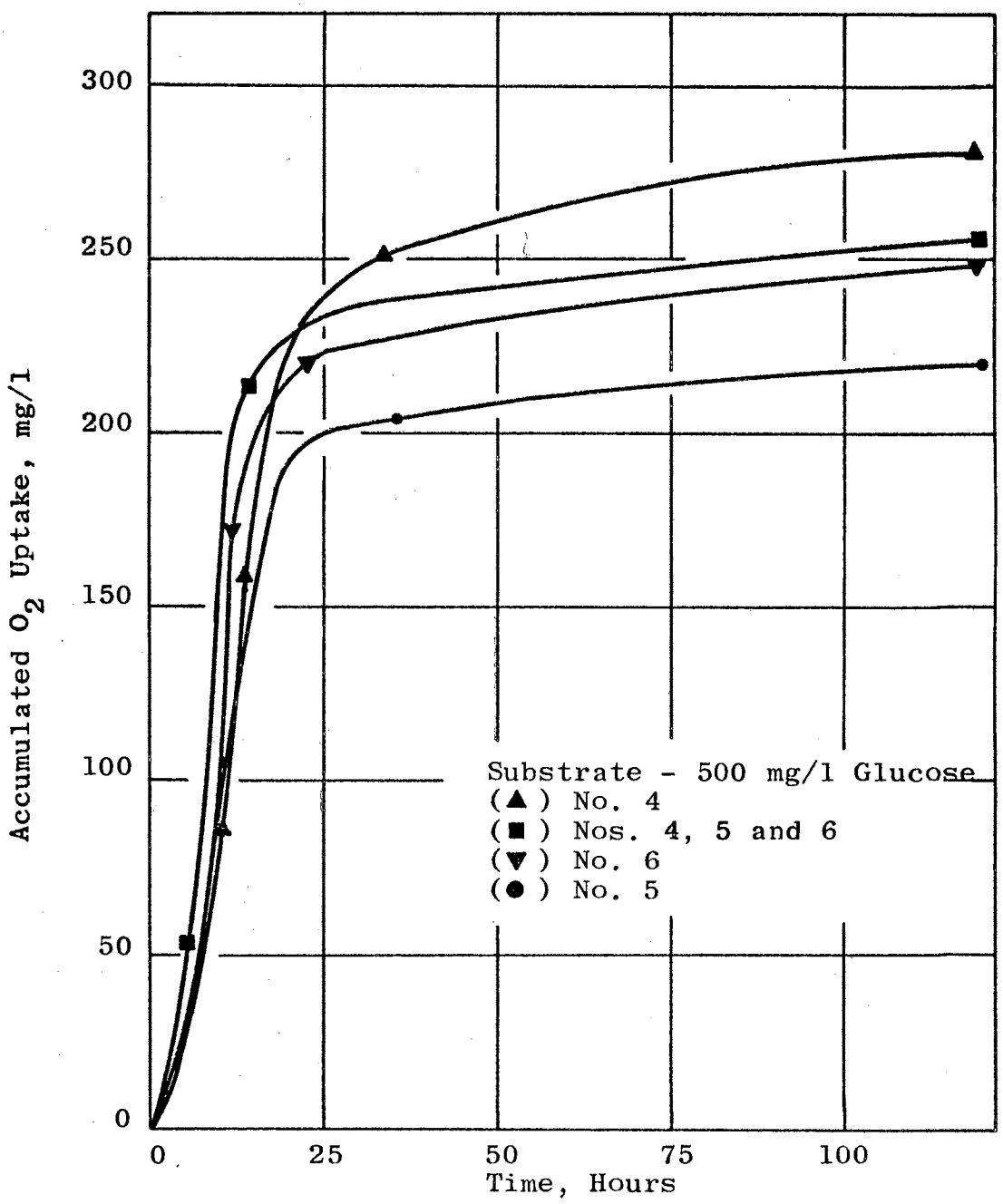


Fig. 26 - WARBURG STUDIES ON PURE CULTURES ISOLATED FROM MIXED POPULATION EXPERIMENTS, USING ACCLIMATED SMALL INITIAL INOCULUM.

In addition, pure cultures were isolated from laboratory batch activated sludge units fed on phthalic acid and glucose respectively. Fourteen pure cultures were obtained from a phthalic acid unit and studied for oxygen uptake characteristics. Oxygen uptake for five representative cultures, from the fourteen, is plotted in Fig. 27. Seventeen pure cultures were isolated from a glucose unit. One out of the seventeen exhibited a bimodal oxygen uptake curve. Fig. 28 shows plots for accumulated oxygen uptake by seven typical pure cultures of the remaining sixteen obtained from the glucose unit. Again, no plateau or secondary rise in oxygen uptake was observed for the results reported in Figures 27 and 28.

The pure culture (No. 13) which exhibited a diphasic oxygen uptake with two phases separated by a plateau was tentatively identified as Escherichia intermedia according to Bergey's Manual (72). The series of characterization tests performed and their results are listed in Table V.

Studies on Pure Culture No. 13

The subsequent results reported in this section are devoted to the investigations conducted with regard to the generality of occurrence and causation for the existence of the plateau in oxygen uptake using pure culture No. 13.

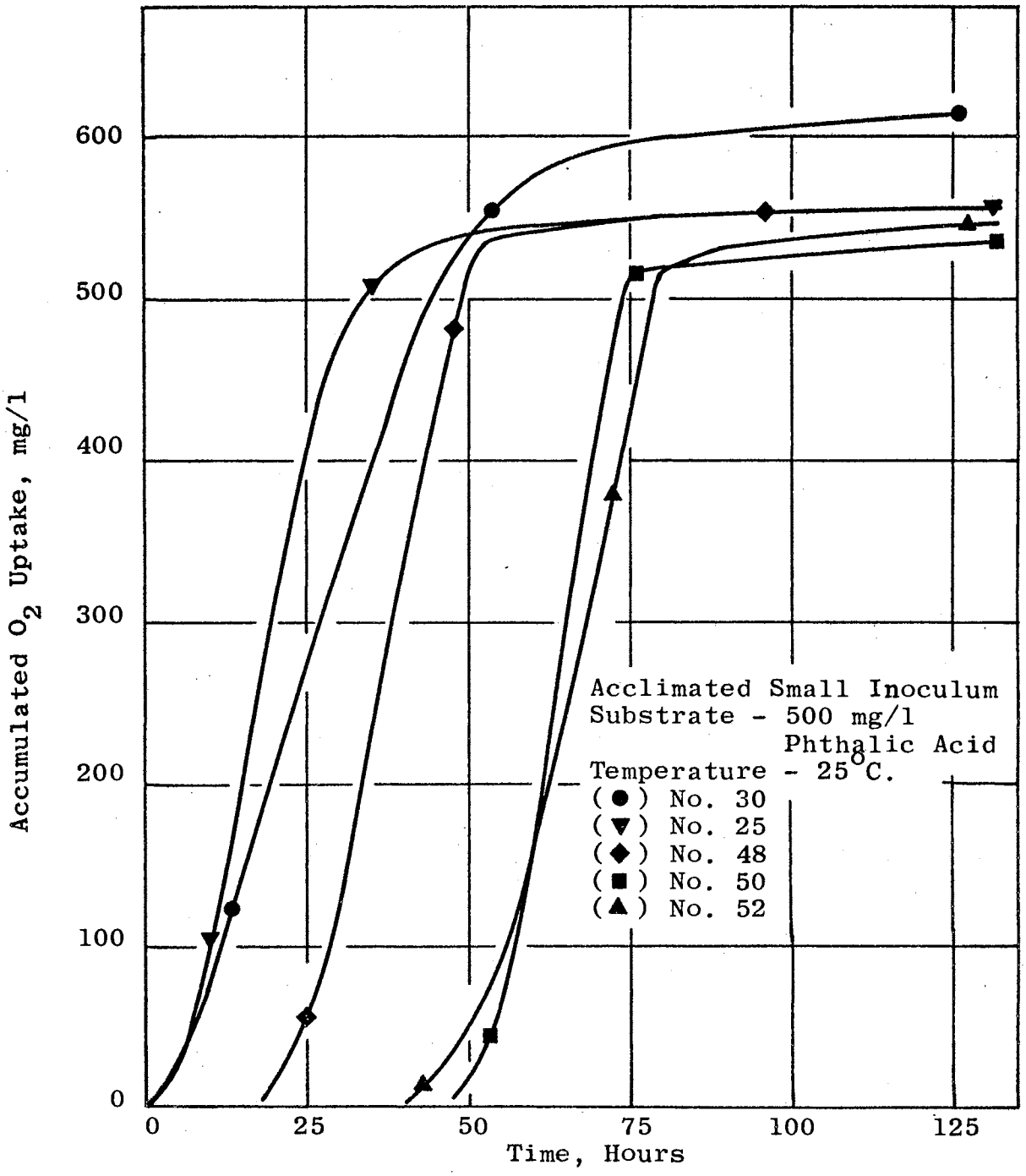


Fig. 27 - WARBURG STUDIES ON PURE CULTURES OF BACTERIA ISOLATED FROM PHTHALIC ACID FED ACTIVATED SLUDGE UNIT.

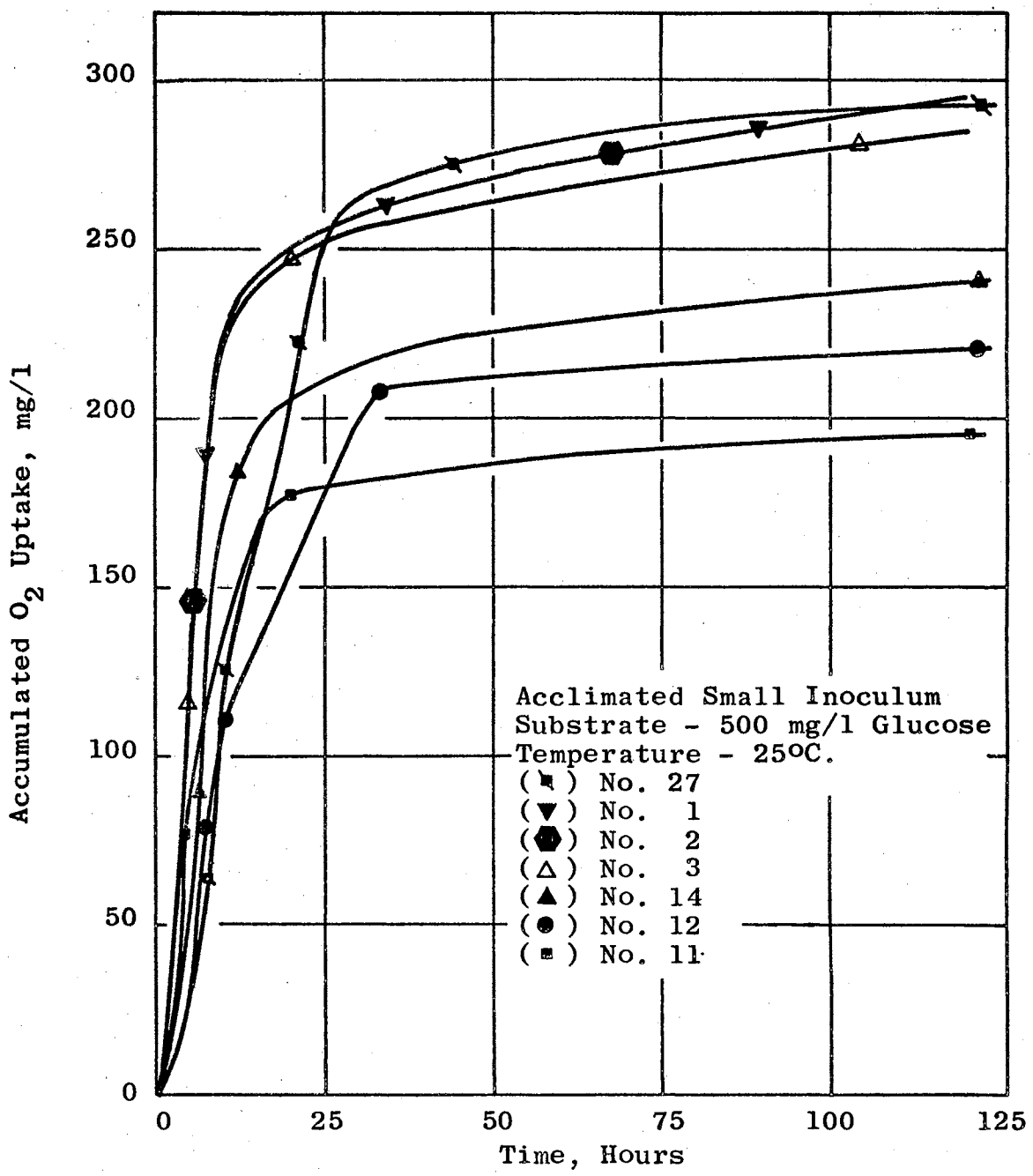


Fig. 28 - WARBURG STUDIES ON PURE CULTURES OF BACTERIA ISOLATED FROM GLUCOSE FED ACTIVATED SLUDGE UNIT.

TABLE V

IDENTIFICATION TESTS PERFORMED ON PURE CULTURE NO. 13

Test Performed	Results
1. Motility in semi-solid agar	+
2. Gram staining	Gram-negative rod
3. H_2S production	-
4. Glucose	Acid and gas +
5. Lactose	Acid and gas +
6. Sucrose	+
7. Indole production	+
8. Acetyl-methyl carbinol formation (Voges-Proskauer test)	-
9. Methyl reduction	+
10. Nitrate reduction to NO_2	+
11. Growth in citrate media	+
12. Starch hydrolysis	+
13. Gelatin hydrolysis	+
14. EMB agar	Metallic sheen

A. Effect of Substrate and Solids Concentrations

Fig. 29 shows plots for oxygen uptake using a small inoculum of acclimated pure culture No. 13. A distinct secondary stage of oxygen uptake was discernible at all concentrations employed; however, it is seen that as the concentrations of glucose increased the transition period between the two oxygen uptake phases extended, and the onset of secondary rise in oxygen uptake was delayed. The initiation of the secondary rise in oxygen uptake occurred at 23.5 to 34.4 per cent of theoretical oxygen demand. The figures in parenthesis indicate the percentage of theoretical oxygen uptake expressed at that point.

Results for accumulated oxygen uptake using initial solids concentrations of 13, 128, 256, 640 and 1280 mg/l of pure culture No. 13 are reported in Fig. 30. Initial glucose concentration was 500 mg/l for all systems in the experiment. While the system containing 13 mg/l initial solids concentration showed two stages of oxygen uptake separated by a plateau, the 128 mg/l solids concentration showed no plateau or secondary rise in oxygen uptake. However, a second stage of oxygen utilization reappeared at 256, 640 and 1280 mg/l initial solids concentrations. Also, it is seen that secondary oxygen uptake in these systems was proportional to the initial solids concentrations. It is interesting to note that the endogenous controls for these systems (i.e., 256, 640 and 1280 mg/l) showed a concomitant

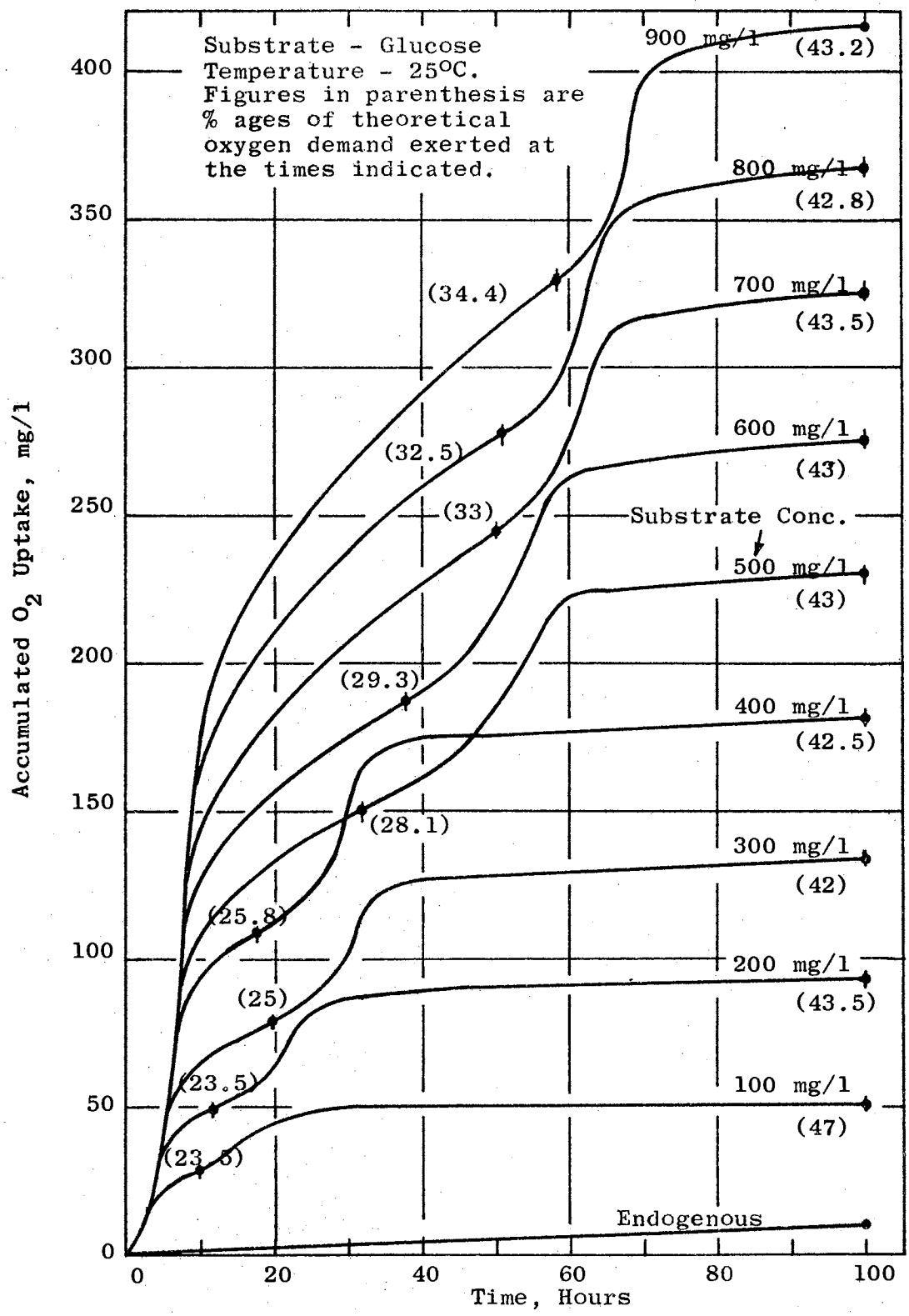


Fig. 29. - WARBURG STUDIES USING SMALL ACCLIMATED INOCULUM OF PURE CULTURE NO. 13.

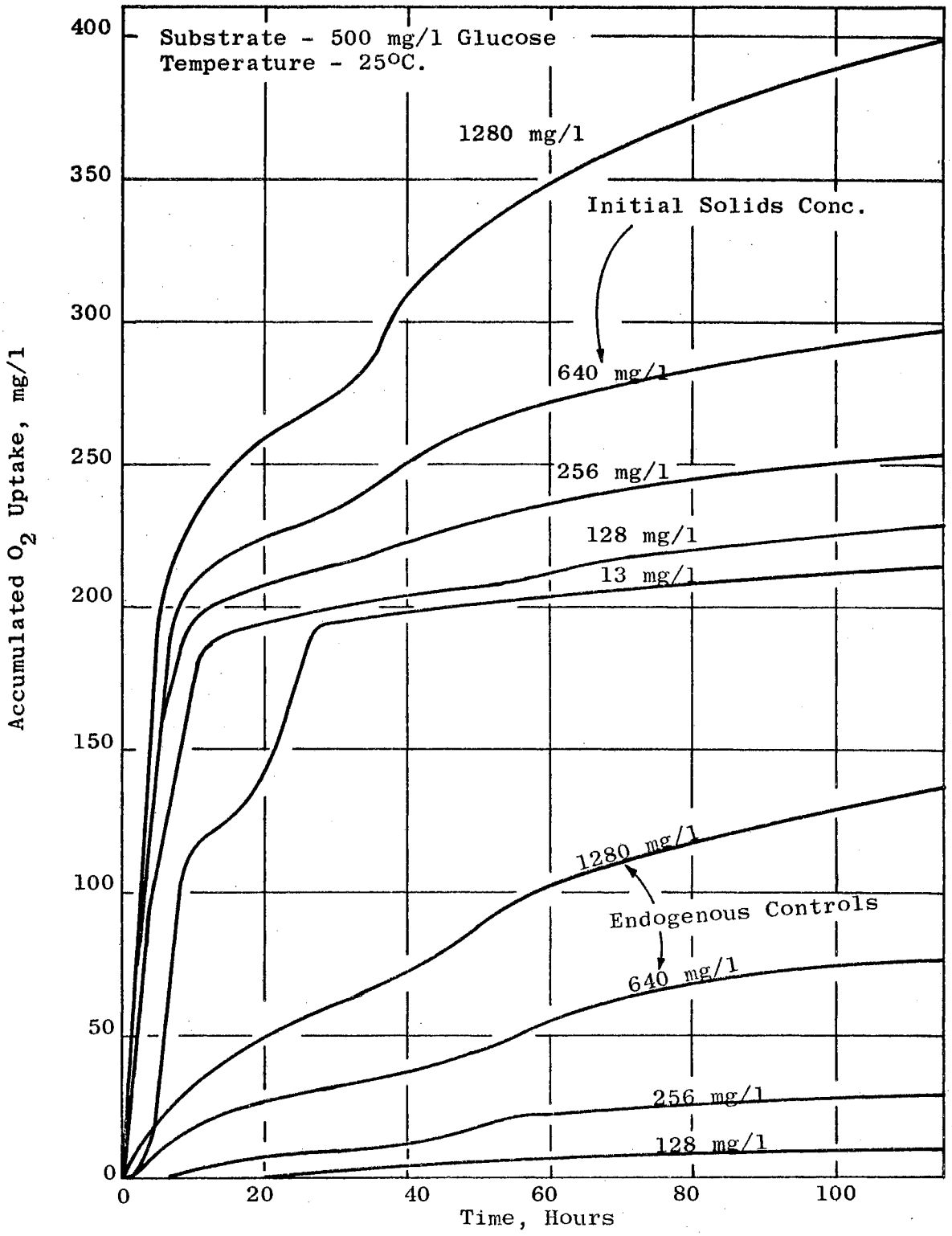


Fig. 30 - WARBURG STUDIES USING VARIOUS INITIAL SOLIDS CONCENTRATIONS OF PURE CULTURE NO. 13.

secondary rise in oxygen uptake. Moreover, the second stage of oxygen uptake in all systems (endogenous and fed) occurred simultaneously and was delayed as compared to the lowest initial solids concentration system (i.e., 13 mg/l). This experiment was performed twice, and the same results were obtained.

B. Oxygen Uptake using Various Compounds

Oxygen uptake values with a variety of substrate solutions inoculated with a small amount of glucose-acclimated pure culture No. 13 are plotted in Figures 31A and 31B. Results using pure culture No. 13 acclimated to the specific substrates are shown in Fig. 32. The various solutions tested consisted of single carbon sources, such as sorbitol, ribose, acetate and glutamate, and complex substrates such as nutrient broth and sterile sewage. On comparing the systems using glucose-acclimated seed the following observations were made:

a) Except for different lag periods the nature of the oxygen uptake curves was the same for the acetate and glutamate systems (Figures 31A and 32). No plateau was observed in the oxygen uptake curves.

b) Similar oxygen utilization curves were expressed for ribose in both cases. A slight break in oxygen uptake was noted in both Figures 31A and 32, but the break was not pronounced.

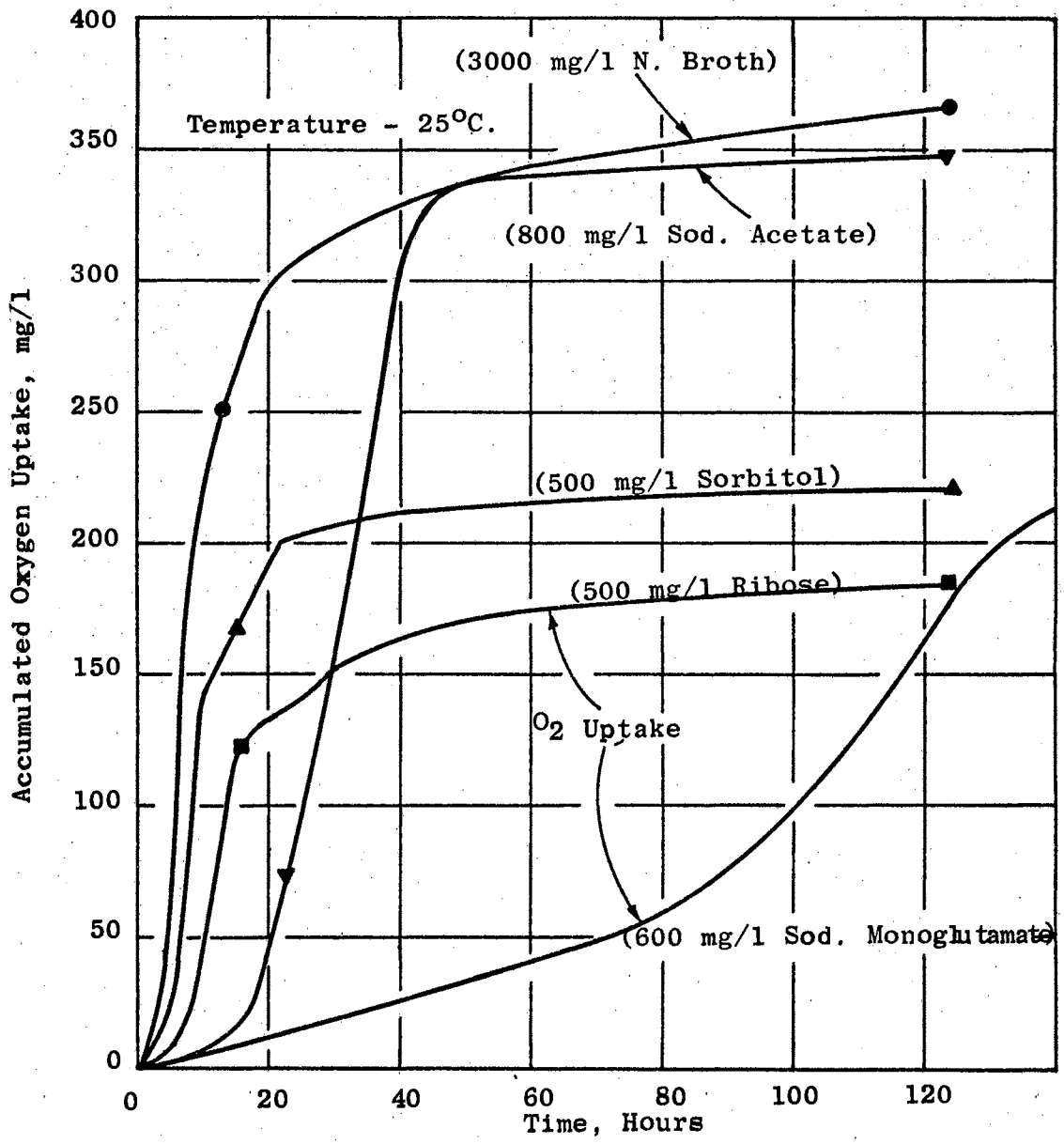


Fig. 31A - WARBURG STUDIES ON VARIOUS COMPOUNDS USING SMALL INOCULUM OF GLUCOSE-GROWN PURE CULTURE NO. 13.

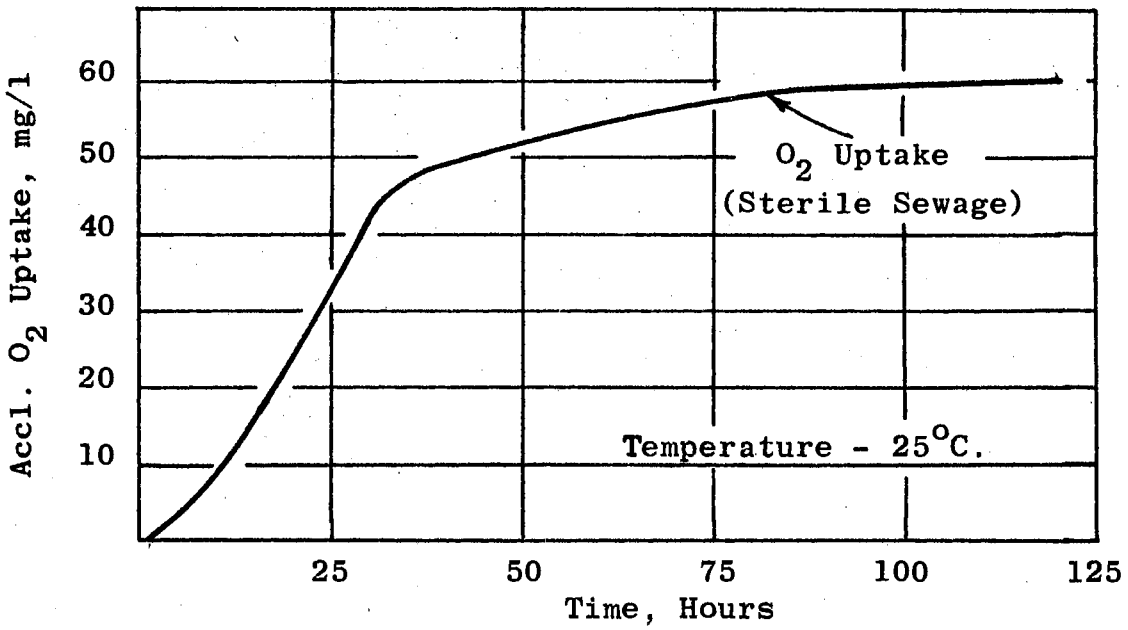


Fig. 31B - WARBURG STUDIES ON VARIOUS COMPOUNDS USING SMALL INOCULUM OF GLUCOSE-GROWN PURE CULTURE NO. 13.

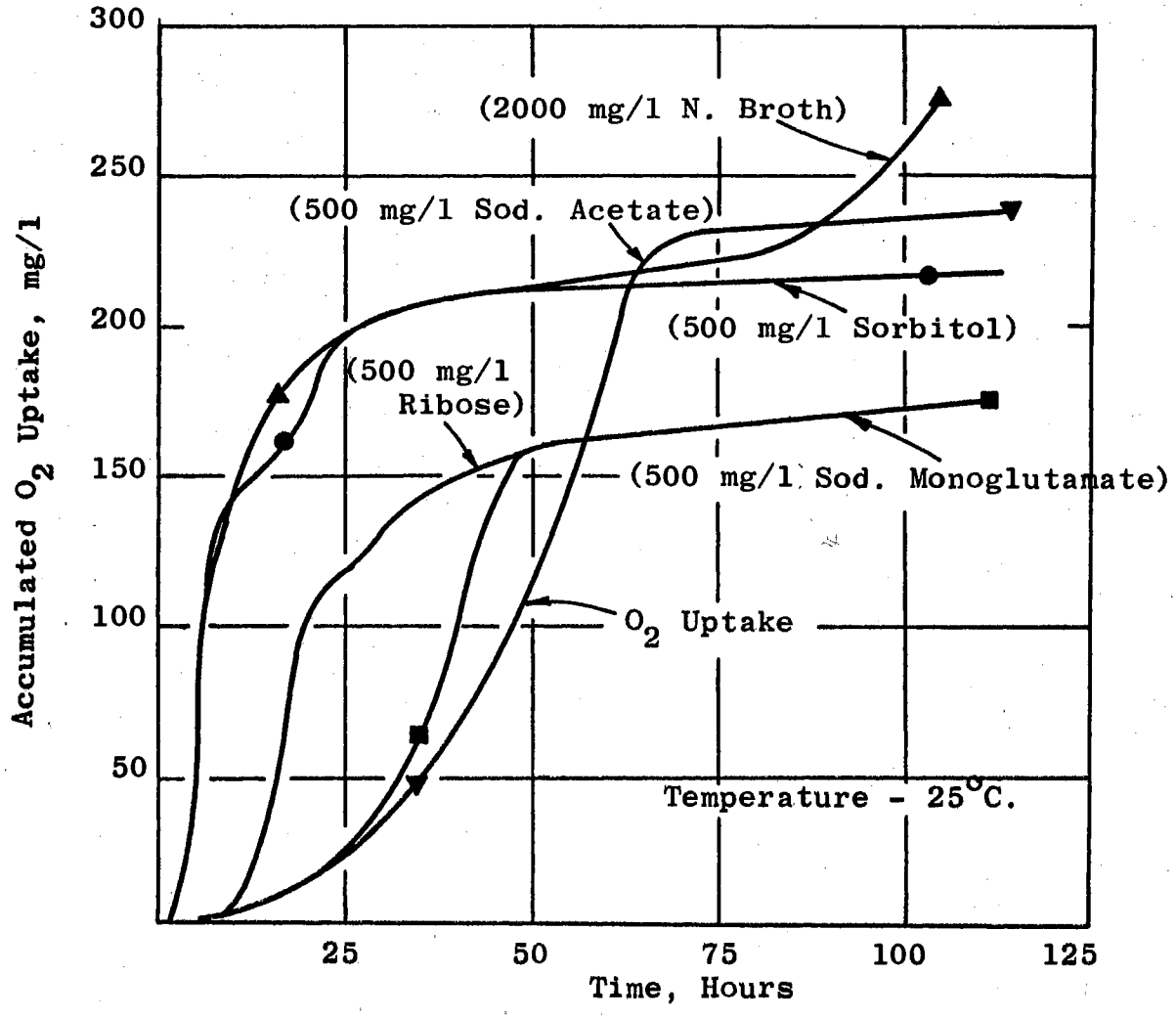


Fig. 32 - WARBURG STUDIES ON VARIOUS COMPOUNDS USING SMALL INOCULUM OF SUBSTRATE ACCLIMATED PURE CULTURE NO. 13.

c) While the sorbitol system in Fig. 32 showed a two-stage oxygen uptake curve, with the two stages separated by a period of low activity, the oxygen uptake expression for the same systems in Fig. 31A exhibited two distinct rates during the rapid oxygen utilization phase but without a pause.

d) No secondary rise in oxygen absorption was discerned for the nutrient broth using glucose-acclimated seed (Fig. 31A); however, a late rise in oxygen uptake was observed for the substrate-acclimated seed system (Fig. 32).

e) The oxygen utilization pattern using sterile sewage did not exhibit a plateau (Fig. 31B).

The studies on pure culture No. 13 thus far presented indicate that a plateau occurs at all concentrations of glucose. Initial solids concentration has a varied effect on the occurrence of the plateau and the second stage of oxygen uptake. At high solids concentrations the second stage of oxygen uptake, when it occurs, is proportional to the initial solids concentration. However, the occurrence of diphasic oxygen uptake with a plateau between the two phases is not invariable and is a selective response dependent on the substrate. Except for the nutrient broth system the initial acclimation of seed to the respective substrates did not significantly affect the oxygen utilization patterns.

C. Effect of Chloramphenicol on Oxygen Uptake

An inhibitor of protein synthesis was employed to examine the possible involvement of a readjustment of the enzyme system(s) between the two stages of oxygen exertion by the pure culture No. 13. It was intended to block protein synthesis in the system at various metabolic levels by treatment with chloramphenicol and to study its effect on oxygen uptake.

Chloramphenicol was tipped from the side arms into the main reaction chamber of the Warburg Flasks A, B and C (see arrows Fig. 33) to give a final concentration of 50 mg/l in the flasks. Fig. 33 shows modifications in the oxygen consumption after tipping the chloramphenicol into Flasks A, B and C as compared to the control system. The oxygen utilization by Flask A, in which the chloramphenicol was added well before the time of maximum glucose removal, decreased sharply as compared to the control system at about the point of maximum glucose removal, i.e., 9 hours. Subsequently very little oxygen (approximately 10 mg/l) was utilized by the cells in Flask A. The oxygen uptake in Flask B, into which the inhibitor was tipped 1.5 hours after the point of glucose exhaustion, also decreased as compared to the control flask curve and increased only half as much as the control from the point of tipping until the end of the experiment. While the viable bacterial count in Flask B dropped a little at the point of addition of chlor-

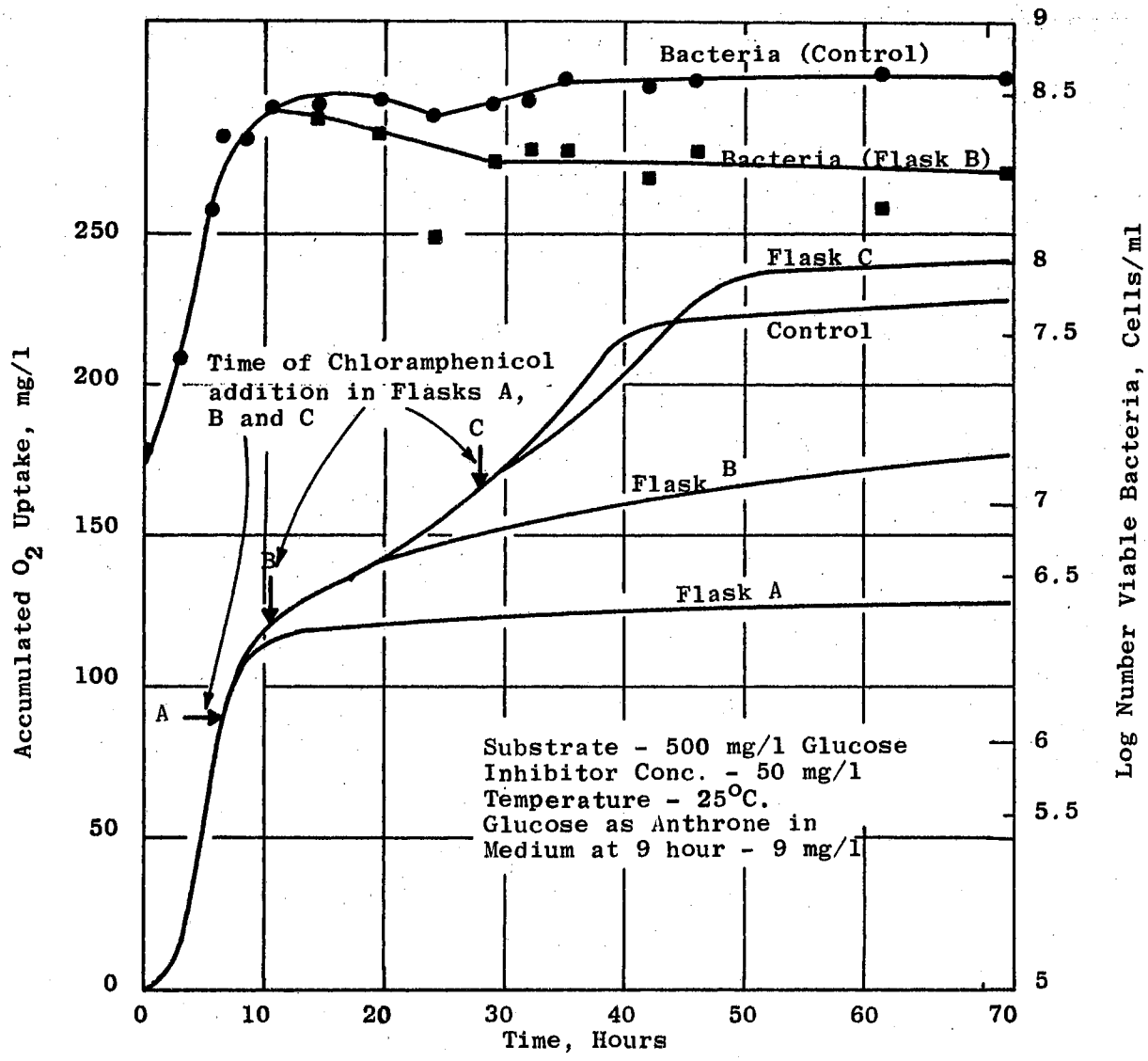


Fig. 33 - EFFECT OF PROTEIN SYNTHESIS INHIBITOR CHLORAMPHENICOL ON O_2 UPTAKE AND CELL COUNT USING PURE CULTURE NO. 13.

amphenicol and then remained constant, the control system showed slightly larger numbers. The addition of chloramphenicol to Flask C when the secondary rise in oxygen uptake was well under way caused the oxygen uptake in Flask C to lag that in the control; however, at the end of the experiment Flask C had consumed 14 mg/l more oxygen than the control.

D. Changes in System Parameters during Long Term Aeration Studies

Fig. 34 shows variations in biochemical parameters for a three-day Warburg experiment. It is noted that the maximum glucose removal, measured by the anthrone test, corresponded to the break in the oxygen uptake curve. The COD removal curve closely followed the oxygen uptake pattern. Although all glucose was exhausted from the system, only half of the COD was removed. However, a maximum of 130 mg/l of volatile acids, as acetic acid, was found in the medium near the beginning of the second stage of oxygen uptake. Volatile acid rapidly disappeared during the secondary rise in oxygen uptake and was nearly exhausted at the end of the second stage of O_2 utilization. A residual COD of 100 mg/l persisted in the medium.

Cellular protein content reached a peak corresponding to the point of maximum volatile acids in the medium, and attained another peak at the end of the second stage of the

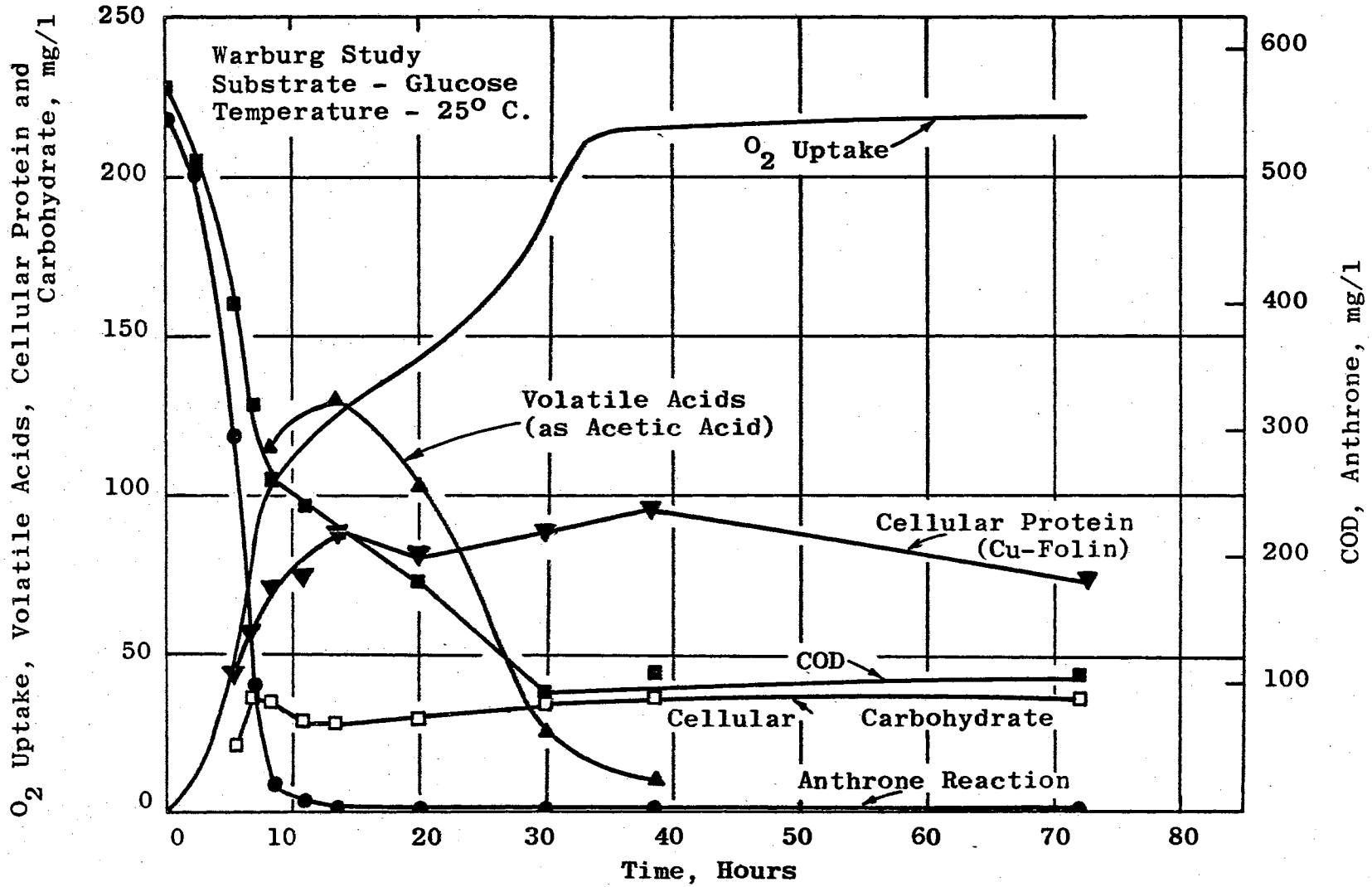
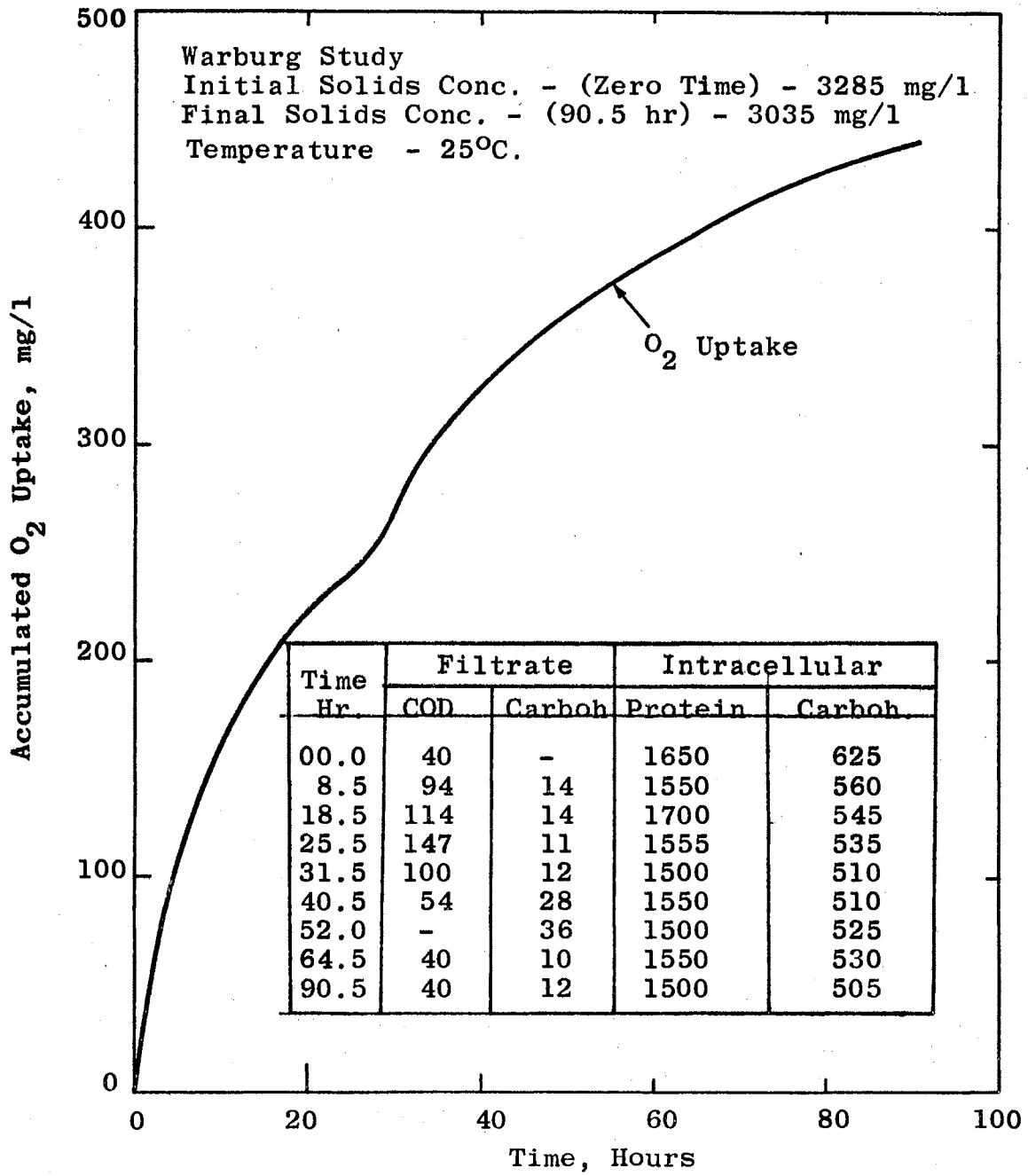


Fig. 34 - LONG TERM AERATION STUDY USING SMALL INOCULUM OF PURE CULTURE NO. 13.

oxygen uptake. A very small drop in cellular protein was noticed in the later part of the experiment. Cell carbohydrate content attained a small peak much earlier, corresponding to the maximum glucose removal, and showed a small rise during the second stage of oxygen utilization.

E. Endogenous Metabolism of Glucose-grown Cells

This study was undertaken to gain some insight into the reappearance of the secondary rise in oxygen uptake in high initial solids concentration systems (see Fig. 30). Since the endogenous controls for the same systems (Fig. 30) also showed a diphasic oxygen utilization, it was felt that the study of endogenous metabolism of the cells would be a very fruitful approach in helping to reveal the biochemical reactions leading to such a phenomenon. The parameters studied during endogenous metabolism are shown in Fig. 35. Diphasic oxygen uptake was observed in the absence of external substrate. An examination of data revealed that portions of cellular carbohydrate and protein were metabolized by the end of the first stage of oxygen uptake. Whether there was a substantial protein degradation during this period could not be rigidly determined from the nature of data obtained; however, it is seen that there was a buildup of COD-responsive, noncarbohydrate material in the medium until the end of the first stage of oxygen uptake. This material disappeared during the secondary oxygen up-



Time Hr.	Filtrate		Intracellular	
	COD	Carboh	Protein	Carboh.
00.0	40	-	1650	625
8.5	94	14	1550	560
18.5	114	14	1700	545
25.5	147	11	1555	535
31.5	100	12	1500	510
40.5	54	28	1550	510
52.0	-	36	1500	525
64.5	40	10	1550	530
90.5	40	12	1500	505

Fig. 35 - STUDY OF ENDOGENOUS METABOLISM OF GLUCOSE-GROWN PURE CULTURE NO. 13. QUANTITIES GIVEN IN THE TABLE ARE IN mg/l.

take. A loss of 250 mg/l of cells was recorded at the end of the experiment.

PHASE III - Phasic Oxygen Uptake in Multicomponent Substrate Systems

BOD Studies in Multicomponent Systems

The course of substrate removal during the exertion of BOD in a two-component carbon source (glucose-sorbitol) medium, using glucose-acclimated and sorbitol-acclimated seeds, is plotted in Figures 36 and 37, respectively.

In Fig. 36 it is seen that the utilization of glucose and sorbitol was phasic. The sequential metabolism of these substrate components was clearly reflected in the BOD curve resulting in a diphasic oxygen uptake separated by a pause or plateau. The first cycle of oxygen uptake corresponded to the glucose removal and the second cycle of oxygen utilization corresponded to sorbitol utilization. In Fig. 37, using sorbitol-acclimated seed, glucose and a part of the sorbitol were utilized almost concurrently. The remaining sorbitol in the system was removed at a very slow rate. No phasic phenomenon was observed in the BOD expression.

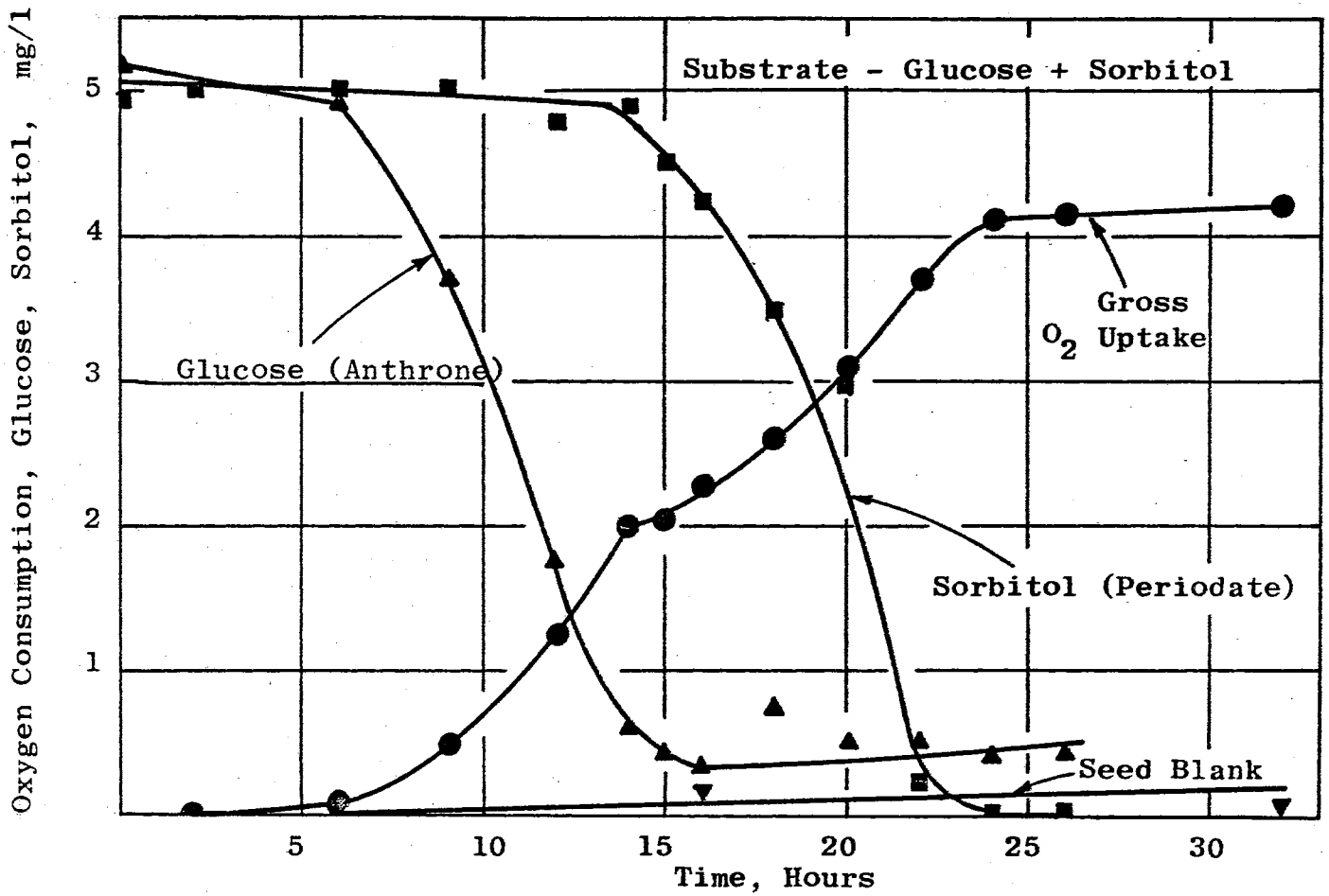


Fig. 36 - BOD EXERTION AND SUBSTRATE REMOVAL STUDIES IN THE BOD BOTTLE, USING GLUCOSE ACCLIMATED SEED

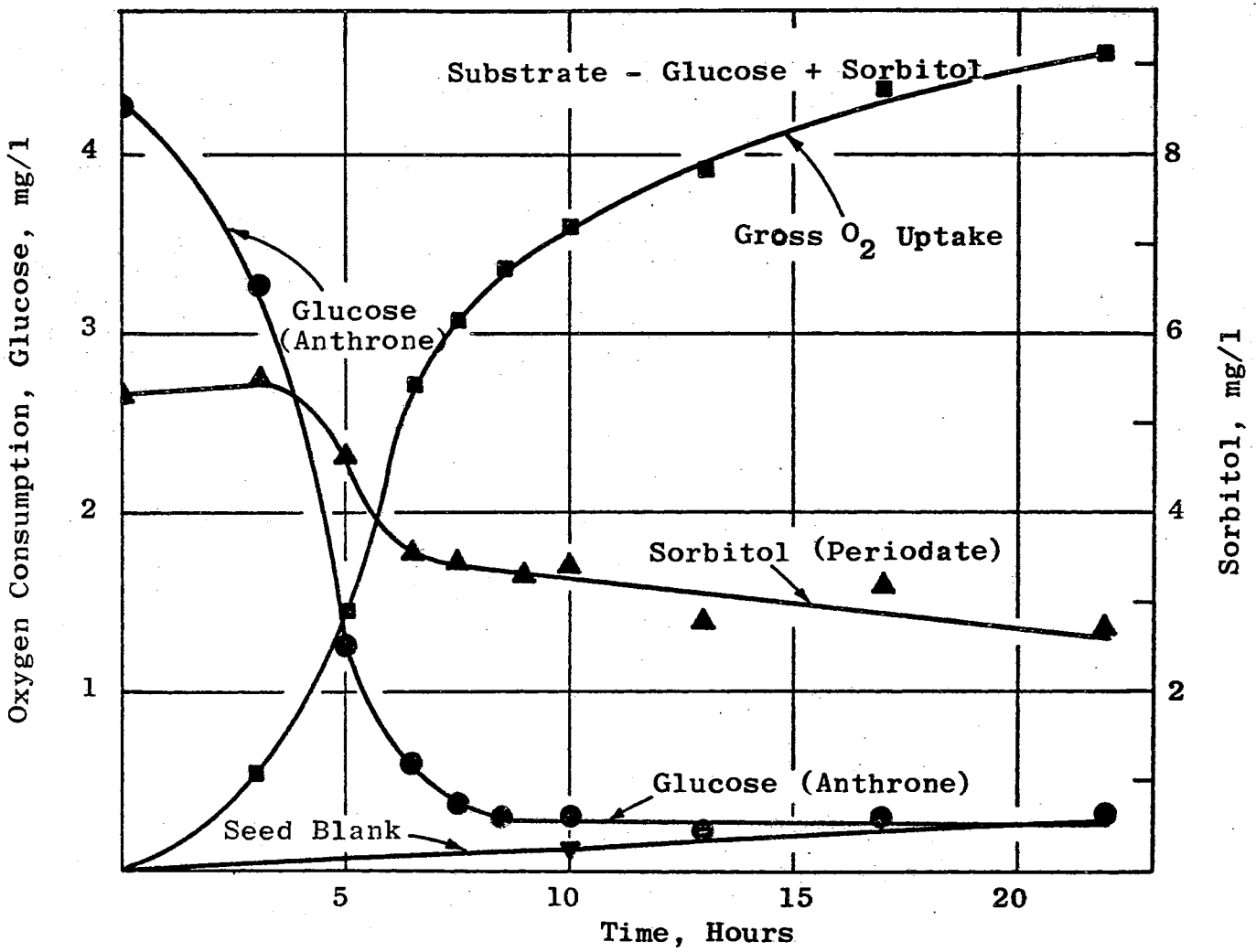


Fig. 37 - BOD EXERTION AND SUBSTRATE REMOVAL STUDIES IN THE BOD BOTTLE USING SORBITOL ACCLIMATED SEED.

DISCUSSION

PHASE I

The results presented in Phase I clearly delineate the role of the mechanism of Theory 1, i.e., changes in predominance from bacteria to protozoa, in causing a plateau and the second stage of oxygen uptake in both low and high energy batch systems using heterogeneous populations. The explanation proposed in Theory 3, involving intracellular substrate(s) utilization following enzyme(s) synthesis in generating a plateau in oxygen uptake has been demonstrated to be rather unimportant for heterogeneous populations as revealed by the chloramphenicol studies (Figures 21 and 22). By the same token, additional evidence is available for substantiating the low probability that the mechanisms outlined in Theories 2 and 4 cause the plateau in heterogeneous population systems. In addition, since chloramphenicol completely prevented bacterial growth in the flasks immediately after its introduction, the nature of the results shown in Figures 21 and 22 lead to the conclusion that a secondary predominance in the bacterial population (part of Theory 1) cannot be held responsible for the causation of the plateau and the second phase of oxygen uptake.

Based on these investigations it is now feasible to correlate the exertion of biochemical oxygen demand during waste purification to population variations in the biomass in batch systems employing mixed biological populations such as are found in sewage. In a typical two-phase oxygen uptake curve, with the two phases well separated by a plateau, the first phase of rapid oxygen uptake is associated almost exclusively with the bacterial metabolism of the extracellular carbon source and the readily available intracellular substrates. During this phase cells respire part of the substrate to provide energy for anabolic processes. The bacterial population increases and reaches a limiting value, depending upon the environmental conditions and available substrate. Following the exhaustion of substrate, the cells start to respire the readily available cellular substance such as carbohydrate and protein. The contribution of protozoan activity to the oxygen uptake during this period is negligible. Protozoa mainly use bacteria as food, but the possibility of direct carbon source metabolism by protozoa cannot be ignored. Butterfield et al. and Pillai and Subrahmanyan have reported direct carbon source utilization by some predators found in activated sludge at substrate concentrations of the order of those found in sewage (44)(50). Howver, in the dilute systems existing in the BOD bottle the subsistence of protozoa would seem to depend only upon the availability of

bacteria for food (44). In any event the bacteria multiply much faster than the protozoa and the latter, being present in much smaller number, do not aid significantly in waste water purification in the first phase of oxygen uptake.

The plateau or the pause between the two stages of oxygen uptake represents the period of endogenous bacterial metabolism which, if continued in the absence of protozoa, will not consume any considerable amount of oxygen. Correspondingly, the bacterial count during this time lingers in the range of maximum population already attained.

After some time, depending upon the initial protozoan population in the seed material, the multiplying protozoa reach a level at which their contribution to oxygen uptake becomes noticeable. This results in the appearance of a secondary rise in the oxygen uptake. The second stage of the oxygen demand is exerted totally through the proliferation processes of protozoa feeding on bacteria. Thus the period of the second phase of oxygen uptake corresponds to a continuing decline in the viable bacteria count. The secondary oxygen uptake is expressed as an autocatalytic type curve due to the growth of protozoa from initially small numbers. The above statement is borne out by the results shown in Figures 15, 16, 19 and 20. It is observed that nearly half of the second stage oxygen uptake has been satisfied when the protozoan population attains a peak. The remaining half is exerted as the slowly declining pro-

tozoan population metabolizes bacteria until the system comes into balance with respect to the protozoan and bacterial population.

The general sequence of degradation of intracellular materials responsible for the oxygen uptake immediately beyond the point of total substrate removal appears to be carbohydrate, then protein. Gaudy and Englebrecht, and McWhorter and Heukelekian have demonstrated depletion of cellular carbohydrate immediately after substrate exhaustion and before any loss in cellular protein in proliferating systems (73)(39). The results reported in Fig. 18 reveal that a certain portion of cellular protein serves as a readily available substrate for oxidation, following which no decrease in the protein content is observed in the absence of protozoa even after an extended period of aeration. Also, a very small reduction in the dry weight of the cell mass is noted after the utilization of this portion of cellular protein, whereas in the presence of protozoa (Fig. 17) the cellular protein is reduced to a very small amount and a great reduction in the solids content is observed resulting in a high degree of clarification. A rise in the carbohydrate content of the filtrate was discerned beyond the plateau in the biochemical response study using sewage seed (Fig. 17). Bhatla and Gaudy have observed similar increases in the COD and anthrone values of the filtrate during the later portion

of secondary oxygen uptake using sewage and acclimated seeds (7). The explanation for such a result is probably the excretion of bacterial components, for example, cell wall constituents, into the medium as end products of the metabolism of predators feeding on the bacteria. The absence of the rise in carbohydrate in the filtrate in Fig. 18, when no protozoa are present, substantiates this explanation.

The net oxygen uptake curve described by a heterogeneous population in a discontinuous (batch) system is a summation of the relative oxygen utilization curves due to bacterial and protozoan activities; hence, the length of the plateau is a function of the relative timing of growth of the bacteria and protozoa in the system. A long plateau is created in the oxygen uptake curve when the protozoan growth lags considerably the bacterial growth, i.e., the occurrence of maximum numbers for bacteria and protozoa are very much apart on the time scale (Figures 10 and 11). The plateau is completely masked when the bacteria and protozoa attain maximum population almost concurrently (Fig. 9). A shorter pause is generated for intermediate situations of relative bacterial and protozoan growth patterns. This is substantiated by results shown in Figures 7, 8 and 12. In other words, the implication is that the predator-bacteria ratio in the seed is of vital importance in determining the severity of the pause between the two stages of oxygen uptake. This point is

demonstrated in the results presented in Fig. 13. Reduction of the initial protozoan population by five and ten times in the seed yielded an increasing length of the plateau, but 100 and 200 fold dilution of the sewage seed apparently diluted out all the protozoa in the seed, resulting in an infinite length of plateau, i.e., no second stage of oxygen uptake during the time of the experiment. The effect of the protozoa-bacteria ratio in the inoculum on the length of the plateau was also shown in dilute systems (Fig. 14).

Based on the above understanding of the mechanism of the occurrence of the plateau in oxygen uptake, the results reported by Bhatla and Gaudy regarding the effect of substrate and initial solids concentrations on the length and the time of the occurrence of the plateau (see theoretical concepts) can be analyzed as follows:

a) Effect of substrate concentration using small initial inoculum.

With a small fixed number of protozoa initially present in the seed a definite time period is required for adequate protozoan growth to register a significant amount of respiration activity. Thus the initiation time for the second stage of oxygen uptake is more or less fixed, but when a higher substrate concentration is placed in a similarly seeded system the time for the active bacterial oxygen utilization, i.e., the duration of the first phase of oxygen uptake, is increased, tending to shorten the plateau. This

results in merging of the two phases of oxygen uptake with increasing substrate concentration.

b) Effect of initial solids concentration.

Firstly, the use of higher initial solids concentration also increases the initial numbers of protozoa placed in the system in the same proportions as the increase in the size of the inoculum. This reduces the time to the beginning of the second stage of oxygen uptake, resulting in its early appearance or a shortening and early occurrence of the plateau. Secondly, higher initial solids concentrations increase the duration of active oxygen uptake during the first stage in the sense that a longer time is needed for the endogenous bacterial respiration to fall to a small value. This dual effect of increasing the initial solids concentration tends both to compress the plateau and to cause an early occurrence of the plateau in the net oxygen uptake. The secondary rise in oxygen utilization often observed in the endogenous controls is due to the beginning of predator activity in excess of the bacterial respiration.

While it is implied here that the excess oxygen demand exerted during the purification process upon the addition of protozoa to the bacterial system is due to the metabolism of protozoa using bacteria as substrate, the works reported in the literature do not agree with this conclusion. The term "purification" as herein used is considered to mean chemical purification, i.e., BOD removal as well as

the destruction of the biomass (clarification). Protozoa have been regarded as enhancing purification by aiding in continuously lowering the bacterial number below some limiting value, thereby stimulating the bacterial multiplication responsible for the intensified purification. This theory was proposed by Butterfield et al., and was shared by Phelps, Zehnpfenning et al., and Javornicky et al. (44) (21)(29)(51). These investigators regarded protozoa as having an indirect rather than a direct function in oxygen utilization in the sense that the increased oxygen uptake (more purification) caused by the addition of predators was considered to be contributed by the physiological activities of the bacteria rather than the protozoa. This interpretation made by Butterfield et al. was based on results obtained in dilute systems (the BOD bottle) in which the oxygen uptake was measured as a criterion for purification. Higher oxygen uptake meant greater purification. According to Butterfield et al. substrate removal took place during the entire course of oxygen uptake, and continuous destruction of bacteria by the predators maintained an optimum ratio of bacteria to food. It was reported that the protozoan population itself consumed very little oxygen during growth in a concentrated carbon source system and did not flourish at all in dilute systems in the absence of bacteria. Bacteria were described as "condensers" or "concentrators" of food, and their metabolism by

protozoa was assumed to be essentially the same as the protozoa growing in the concentrated carbon source system. Phelps accepted the concepts of Butterfield et al. (44). He did not present any experimental data when he recognized this mode of action of protozoa in the purification process.

The hypothesis of Butterfield et al. apparently misled Zhenpfennig et al. when they proposed that the BOD reaction occurs in two stages. According to them the first stage of oxygen uptake was controlled by the substrate concentration and proceeded according to the first order reaction kinetics. They felt that zero order kinetics would prevail in the second oxygen uptake phase, since they thought that the O_2 uptake in this phase depended upon the reproduction rate of the bacteria which were continuously multiplying after being continuously destroyed by protozoa. They failed to recognize that the second phase of oxygen uptake is a direct function of protozoan metabolism and that all the organic material available to the bacteria has already been assimilated during the first phase of oxygen uptake.

Javornicky and Prokesova agreed with the theory of Butterfield et al. because they obtained results similar to those of the latter authors (51). However, the results presented by these authors could not be used to test the theory of Butterfield et al. since the investigation was designed to determine the influence of protozoa on the BOD test and only oxygen uptake data was available in addition to bacterial and protozoan population dynamics.

The role of protozoa in the purification process proposed by Butterfield, Purdy and Theriault is herein supplanted, based on the following findings (44):

i) Total substrate removal as anthrone (see Figures 11 and 17) was observed at the beginning of the second stage of oxygen uptake, and no second stage of oxygen uptake was observed in the absence of predator activity. Substrate exhaustion at the end of the first phase of oxygen uptake has also been shown by Bhatla and Gaudy, Grady and Busch, and McWhorter and Heukelekian (7)(74)(39). Hence there was no possibility of bacterial multiplication, stimulated by predator activity, in the complete absence of substrate during the second stage of oxygen uptake. In other words, the increased purification measured as oxygen uptake due to the presence of protozoa in the bacterial system can be ascribed only to metabolism of bacterial cells by predators.

ii) The data on oxygen utilization in Figures 19 and 20 and the excess purification (clarification in this case) obtained by the addition of protozoa to the Serratia marcescens cell suspension again suggested a direct role of protozoan metabolism as responsible for the oxygen uptake in the second stage and the enhanced purification. Bacterial replication could not be held responsible for this increased purification, due to the absence of an external substrate.

iii) Finally, chloramphenicol, which completely arrested bacterial growth in the system before the start of the second stage of oxygen uptake, did not inhibit the oxygen uptake (Figures 21 and 22) during the second stage. This negated the participation of bacterial activity in the second stage of oxygen uptake, indicating an autonomous role of protozoa in the purification process.

These findings lead to the conclusion that in the total purification process protozoa are not accessories to intensified purification by bacteria as suggested by Butterfield et al. but occupy an independent role in completing the purification process. It is realized that in discontinuous systems purification is a phasic phenomenon; the first phase of BOD removal is accomplished mainly by bacteria, and the second phase, consisting mainly of destruction of the biomass (clarification), is attained through the action of predators. While the role of protozoa in the production of clarified effluents in the activated sludge system has been recognized by many workers, Pillai and Subramanyan regarded protozoa as primary agents for total purification (45)(46)(47)(75)(50). The confusion about the role of protozoa in the purification process stemmed from the tendency to interpret the BOD test in terms of substrate removal while it is in fact intended to measure the pollutional characteristics of the waste in terms of oxygen uptake.

The influence of protozoa on the 5-day BOD test is very significant since the present studies show that under normal seed conditions protozoan action usually contributes about 30 per cent of the total 5-day BOD. Obviously, the 5-day BOD test cannot be interpreted in terms of the biochemical oxidation characteristics of bacteria alone. Furthermore, one cannot ascribe any simple kinetic model to the BOD reaction since it is a complex of two growth cycles, i.e., bacterial and protozoan, always relatively displaced, depending upon the character of the seeding material. It is desirable to point out here that the amount of the oxygen uptake exerted during the second stage depends, apart from the quantity of food (the bacterial cells), upon the types of protozoa present. During these studies it was noticed that the small flagellate protozoa which are passed by the 5 μ filter were not very effective in exerting oxygen uptake. However, most of the protozoa responsible for the second stage of oxygen uptake could be passed through a 10 μ filter. The reduction in the secondary oxygen uptake in Figures 14 and 15 using a reduced protozoan population in the seed is most probably due to the selection of protozoan species in the seed upon dilution. It may be added here that different species of bacteria might exhibit different degrees of availability as food for protozoa (44)(51).

Realizing the part played by the predators during the so-called phase of "endogenous" sludge metabolism, present

concepts of the endogenous activity of the sludge are distorted, because most of the oxygen uptake during the so-called endogenous respiration of sludge is actually contributed by the exogenous metabolism of the protozoa using bacteria for food. None of the available definitions for endogenous respiration of sludge used in sanitary engineering literature truly describe the situation. Wuhrmann proposed that respiration of sludge (i.e., washed sludge suspended in suitable buffer solution) can be used as a parameter to predict the capacity of the sludge for waste assimilation (76). In developing this concept Wuhrmann tried indirectly to quantitize the number of cells which would take part in active metabolism of the waste by measuring their respiration rate in the absence of substrate. The theoretical significance of this procedure is lessened when the respiration measured is due primarily to the predators. However, an empirical correlation may still be observed, in some cases, between the sludge's ability to remove substrate and its respiration rate.

PHASE II

One of the main points emphasized by the present research is that there are not many commonly occurring pure cultures of bacteria which exhibit a bimodal oxygen uptake curve. The bimodal oxygen uptake for pure cultures reported by Halvorson, Wilson and Harrison, Marion and Malaney, and Gaudy and Bhatla appear to be rather rare examples (35)(38)(41)(5). The pure culture No. 13 tentatively identified as Escherichia intermedia in the present study was the only one of some sixty pure cultures tested which showed a diphasic oxygen uptake with two phases separated by a plateau. The fact that the diphasic oxygen uptake curve is expressed with this pure culture only with some substrates such as glucose, sorbitol and possibly ribose, points to the specialized nature of the metabolic activities responsible for this phenomenon. Logically, the reason for the absence of a plateau in the oxygen uptake curve with acetate and glutamate as substrates was that metabolism of these compounds did not involve the production of an extracellular catabolite to which acclimation would be required during the normal degradation scheme. However, no explanation is offered for the nonexistence or existence of the secondary oxygen uptake for nutrient broth systems in Figures 31A and 32. It can thus be surmised with considerable certainty that the commonly observed

two-stage oxygen uptake, with a plateau between the stages during BOD exertion using a sewage seed is not generally attributable to a particular metabolic characteristic of the bacteria. Also, there is adequate evidence in Phases I and II with regard to the conclusion that diphasic oxygen uptake is also not expressed due to the interaction of bacterial species present in a mixed bacterial culture.

The general pattern of the utilization of oxygen by a single culture of bacteria or a mixed bacterial culture is described by a phase of rapid oxygen uptake followed by a phase of slow endogenous respiration. From the results of oxygen uptake studies on various pure cultures it was seen that different types of bacteria carry out substrate oxidation to different extents, and their behavior in achieving oxidation in mixture is not predictable through any simple formulation. This observation serves to stress the need for caution against assuming rigid values for the oxidation of particular substrates by heterogeneous populations.

Obviously, Theory 1 could not be called upon to explain the causation of diphasic oxygen uptake observed with pure culture No. 13. From the results presented in Fig. 34 it follows that the first stage of oxygen uptake was due to the incomplete oxidation of glucose to CO_2 and volatile acids. The second stage of oxygen uptake corresponded to the disappearance of the volatile acids accum-

ulated in the medium during the glucose metabolism in the first phase. The utilization of volatile acids during the second stage of oxidation suggests that a new enzyme or enzyme system is induced during the transition or plateau period. This deduction is substantiated by the studies reported in Fig. 33. The blocking of enzyme synthesis, i.e., protein synthesis, by chloramphenicol in Flask A before the point of total glucose removal completely prevented the occurrence of second stage oxidation. However, the addition of the protein synthesis inhibitor 1.5 hours after total glucose removal in Flask B reduced the respiration in the second stage compared to the control. The nearly linear rate of oxygen uptake observed after treatment with chloramphenicol in Flask B indicated that chloramphenicol did not inhibit the metabolism of volatile acids in cells that had already formed some enzyme(s). The modification in the secondary oxygen uptake in Flask C, after tipping in chloramphenicol, is explained as a stimulation of respiration of the cells through interference with growth processes by the inhibitor; this resulted in higher ultimate oxidation. The respiration of the volatile acids was not affected in this case, since the cells possessed a high enough enzyme(s) level for substrate metabolism.

These findings point out that enzyme(s) synthesis is needed for the metabolism of secondary exogenous substrate,

i.e., volatile acids. This mechanism of causation of the plateau and the second stage of oxygen uptake is described by Theory 4 proposed by Gaudy and Bhatla (5). It may be suggested here that the occurrence of this stepwise metabolism of glucose by the culture No. 13 is due to one of the following:

1. Probably the acetate (volatile acids) utilizing system in this bacterium is an induced enzyme system, and formation of this enzyme system is repressed by glucose metabolism. It may also be diluted, largely due to the new growth occurring in the system, especially when a small initial inoculum is used. The fact that the plateau disappeared at the 128 mg/l (see Fig. 30) initial solids concentration can be interpreted as follows: the high initial solids concentration masked the plateau by simultaneous utilization of volatile acids during glucose metabolism due to an incomplete repression of inducible enzyme(s) and smaller dilution of the enzyme system already present in the high initial inoculum.

2. A transport enzyme (a permease) is needed before the acetate can be brought into the cell for utilization, and it is repressed in the presence of glucose. A similar mechanism as in item 1 is suggested for the disappearance of the plateau in higher initial solids concentration system (i.e., 128 mg/l).

It is interesting to note that at high initial solids concentrations (more than 128 mg/l) the secondary oxygen uptake reappeared. This time the second stage of oxygen utilization was proportional to the amount of initial solids used. A similar correlation was reported by Wilson and Harrison, using a pure culture inoculum in phthalic acid (38). Certainly the diphasic oxygen uptake in the high initial solids concentration systems did not resemble the 13 mg/l initial solids system (Fig. 30) since the endogenous controls in the former systems also showed a secondary rise in oxygen uptake. The study of the endogenous metabolism of cells suggested that the first phase of oxygen absorption is attributed to the utilization of storage products such as carbohydrate and protein, while a catabolite from the degradation of these substrates and/or others (not determined in this investigation) accumulated in the medium. This exogenous compound was found to be at least partially responsive to the COD test but mostly noncarbohydrate in nature. The second stage of oxygen uptake was accompanied by the disappearance of the material reactive to the COD test which had continued to build up until the end of the first phase. In general this mechanism may be similar to the one responsible for the occurrence of the plateau in systems with small initial solids concentration (e.g., 13 mg/l), except that the primary substrate in the former case was an intracellular material instead of

the extracellular substrate glucose. Needless to say, this particular aspect needs more detailed investigation to delineate precisely the nature of the products involved; however, in the present investigations it was not intended to study the causation of a plateau in the oxygen uptake expressed by all pure cultures under all conditions. Nevertheless it was essential to study at least one case to find out the cause(s) for the existence of the plateau in pure culture systems. As already stated, diphasic oxygen uptake by pure cultures of bacteria is, according to the results of this study, an uncommon phenomenon; therefore the mechanics for the occurrence of the plateau in culture No. 13 cannot be cited as a general mechanism for the existence of a plateau in heterogeneous populations.

PHASE III

The phenomenon of sequential substrate utilization has been reported by Gaudy, Komolrit and Bhatla, using heterogeneous populations in high energy batch systems comparable to activated sludge systems (59). The present findings demonstrate that this stepwise substrate removal can also take place in a very low energy system such as exists in the BOD bottle; it is therefore proven that there are no essential mechanistic differences between high and low energy systems. In other words, an en masse population response of this type is independent of the substrate concentrations involved.

The results shown in Fig. 36 suggest that sequential substrate utilization may be emphatically reflected in the oxygen uptake curve and may give rise to a brief pause between the two growth cycles, resulting in the appearance of a plateau in the BOD exertion curve. In this case the sequential utilization of exogenous substrates would provide an additional mechanism for the occurrence of a plateau in oxygen uptake. However, it might not be of as great significance as the plateau caused by the predator-bacteria relationship in the seed, due to the brevity of this type of pause. Also, it should be emphasized that a plateau created by sequential substrate removal does not possess the biochemical significance attributed to the commonly observed plateau values, since the location of the plateau in this case depends solely on the ratio of the concentrations of each exogenous substrate, i.e., attainment of the plateau, does not indicate the time of total substrate removal from the system. The role of cell age (an operational parameter defined by Gaudy, Komolrit and Bhatla) in this type of response has been delineated by Gaudy et al. (59). In the case of the BOD experiments one can expect young cell conditions, since it is the practice to use a small sewage inoculum as seed, which allows a new population growth.

SUMMARY AND CONCLUSIONS

The causes for the existence of a plateau in the two-stage oxygen uptake curve related to the BOD test were investigated for heterogeneous population systems and a pure culture system. Oxygen uptake studies were conducted in discontinuous systems with heterogeneous populations on glucose synthetic waste water, using both dilution and manometric techniques, and the bacterial and protozoan growth patterns were followed during the course of oxygen utilization. The effect of protozoa-bacteria ratios in the seed material on the kinetics of the plateau in oxygen uptake was studied. In addition the influence of inoculations of protozoa to bacterial systems on the oxygen demand and other biochemical parameters was investigated. Pasteurization and microfiltration of seed material were used to exclude protozoa from the microbial population used as inoculum. Chloramphenicol was employed to delineate the role of enzyme formation before the secondary rise in oxygen uptake in the generation of a plateau in the oxygen uptake curve.

A series of conditions for the existence of the plateau for a pure bacterial culture (isolated from a

glucose-fed activated sludge unit) was established. The causes for the occurrence of the plateau were investigated along the lines of the theories for the mechanism of its causation proposed earlier by Gaudy and Bhatla. Furthermore, sequential substrate utilization in the BOD bottle using heterogeneous populations was examined and its effect on oxygen uptake was observed.

The results of this research reported in this dissertation support the following conclusions:

1. The diphasic oxygen uptake expression with two phases separated by a plateau, commonly observed in low (the BOD bottle) and high energy (Warburg) systems, is primarily due to changes in predominance in population, i.e., protozoa over bacteria (Theory 1). There is a rare possibility of the observance of this type of kinetics due to the contribution of a secondary bacterial predominance (part of Theory 1) or any of the remaining three proposed mechanisms (Theories 2, 3 and 4) in heterogeneous population systems.

2. The relative population densities of bacteria and protozoa in the initial inoculum are vital in determining the occurrence of the plateau. In general, the length of the plateau may be considered proportional to the relative time lag between the peaks in the bacterial and protozoan populations.

3. The role of protozoa in the total purification process is that of clarification, i.e., destruction of the biomass, and in the absence of predators little or no clarification is possible. In a system comparable to the BOD bottle, purification is achieved in two phases. Bacteria are mainly responsible for the first phase of chemical purification, followed by a second phase of destruction of bacterial cells by protozoa (visible purification).

4. The excess "purification" (i.e., oxygen uptake) attained in the presence of the protozoa is accomplished exclusively through the metabolism of bacteria by the predator (protozoa) population, and is not accomplished by continuous bacterial multiplication stimulated by predators.

5. The influence of protozoa is very significant when oxygen consumption is taken as a parameter of purification such as is done in the BOD test. Under normal seeding conditions during a 5-day BOD test, protozoa are responsible for approximately 30 per cent of the BOD exerted, and the second stage of oxygen uptake after a defined plateau is due totally to the respiration of protozoa feeding on the bacteria.

6. Both quantity and quality of protozoan species are important in determining the degree of clarification and the magnitude of oxygen consumed in excess of the bacterial requirements (i.e., second stage O_2 uptake).

7. Protozoa found in sewage are in general resistant to chloramphenicol up to a concentration of 500 mg/l.

8. Two-phase oxygen utilization with two phases connected by a plateau is not a common characteristic of pure bacterial cultures, and is a fairly rare occurrence. The cause(s) for such an oxygen uptake expression can involve any of the mechanistic theories (2, 3 or 4) or a combination thereof.

9. Under conditions of very small initial inoculum, a pure culture (No. 13, tentatively identified as Escherichia intermedia) exhibited a diphasic oxygen uptake curve including a plateau between the two phases. The occurrence of the plateau was found to be dependent upon the nature of the substrate and the initial solids concentration, but was independent of the substrate concentration.

10. The second phase of O_2 uptake observed with pure culture No. 13 was found to be due to the utilization of volatile acids excreted in the medium during glucose metabolism. A time period needed for the induction of new enzyme(s) for the oxidation of these catabolites produced from glucose appeared as a plateau in the oxygen uptake (Theory 4).

11. Sequential substrate utilization takes place in low energy systems and may create a plateau in the oxygen uptake expression corresponding to the exhaustion of each substrate from the system.

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