THE GROWTH OF *E. COLI* AT VARIOUS PHOSPHATE CONCENTRATIONS

by

D. M. Griffin, Jr.

Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE
in
Sanitary Engineering

APPROVED:

R. C. Hoehn, Chairman

E. M. Jennelle

A. C. Hendricks

August, 1971

Blacksburg, Virginia

ACKNOWLEDGMENTS

The author would like to take this opportunity to express his deep appreciation to the following:

To Dr. R. C. Hoehn, his thesis advisor, for his encouragement, guidance and constructive criticism.

To Dr. Ernest M. Jennelle and Dr. Albert C. Hendricks for their help and suggestions.

To for his valuable laboratory assistance.

To for preparation of the graphs and for typing the manuscript.

This research was supported by an Environmental Protection Agency Traineeship funded under training grant WP 166-03 (SA).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

One of the most serious problems confronting personnel concerned with problems of pollution control at this time is that of accelerated eutrophication, the process by which nuisance algal growths hasten the aging process of certain bodies of water. Associated in part with this accelerated aging process, the affected body of water may become clogged with attached aquatic plants, develop foul odors, and given time, may become a public nuisance.

Although eutrophication is a naturally occurring process, activities of man such as the discharge of certain types of treated and untreated waste into receiving waters have greatly accelerated it. As a result, much research has been initiated in recent years to try to determine the best way to retard and control eutrophication.

Phosphorus has been mentioned by many researchers as being the limiting nutrient in eutrophication. Most agree that concentrations as low as .02 milligrams per liter will support an algal bloom. Waste produced by man and his activities is a significant source of phosphorus that generally is ineffectively removed by routinely employed treatment processes. Control of eutrophication by limiting the phosphorus concentration in wastes discharged into receiving waters will necessarily involve a considerable

monetary expenditure in developing new treatment processes and modifying older ones.

In view of this fact, further research should be conducted to try to determine more precisely the role of phosphorus in eutrophication, especially the role it plays, if any, in the algae-bacteria symbiotic relationship. This relationship has recently been suggested to be of importance by several researchers as having a definite role in the stimulation of large nuisance algal growths.

Objectives

The objective of this research was to demonstrate whether or not bacterial growth could be stimulated by increasing the concentration of phosphorus in the growth media. If such stimulation could be demonstrated a hypothesis might then be formed as to how this stimulation related to the process of eutrophication.

Scope

The research was conducted using a lab-scale, continuous-culture unit and a single bacterium, *E. coli*. Synthetic media containing varying amounts of phosphate were used as substrate, and growth was measured in terms of turbidity increases in the cultures. Oxygen utilization by the bacteria was measured using the Warburg Respirometer to determine whether or not increases in phosphorus

concentration increased bacterial respiration. The amount of oxygen utilized was assumed to equal the amount of carbon dioxide produced by the bacteria.

REVIEW OF THE LITERATURE

When one begins a literature review on such a vast and complex process as eutrophication, he is immediately confronted with an extremely large volume of material, plentiful in some areas, painfully lacking in others. At the present time, however, most authors regard eutrophication as a complex and little-understood process during which algal blooms degrade surface waters by causing their natural and, in some cases, premature aging. This process, given enough time, can convert a valuable natural resource into an odious nuisance.

In the past, many inorganic elements have been investigated as to their effect on algae growth and stimulation. At the present, phosphorus or carbon are thought to be the limiting nutrients in most observed cases of eutrophication. More recent research (15) has indicated that bacteria, existing symbiotically with algae, may also be a factor in algae blooms.

In view of the many complex processes which normally occur in any body of water, it is essential that one obtain as full an understanding as possible of the role of phosphorus and carbon in aquatic ecosystems before attempting to elucidate the mechanisms by which they control algae growth.

Both autotrophic and heterotrophic forms of life must have sources of carbon, nitrogen, and phosphorus in order to proliferate. The ratio of these elements in the individual cell depends on the species of algae or bacteria being considered (10).

Although algae can utilize organic carbon, it is generally believed that they can divide only when inorganic carbon sources are available (15). In addition, there is no evidence that algae are able to utilize their intracellular carbon compounds to support growth (15). In aquatic systems the autotrophic population utilizes carbon dioxide and bicarbonate for growth and releases oxygen to its environment. The heterotrophic population utilizes oxygen in the degradation of organic matter and releases carbon dioxide, completing the cycle.

Several sources of inorganic carbon, other than that from bacteria, have been mentioned as being available to algae. The most significant sources that are mentioned are: 1) the bicarbonate alkalinity system of the water (16), 2) anaerobic fermentation (10), and 3) carbon dioxide diffused from the atmosphere (10). Physical parameters, such as pH, temperature, and light intensity, are also known to affect the availability of inorganic carbon (15).

Research has shown carbon to be a limiting nutrient, not only in algal growth, but also in the growth of higher

plants. Quinn and Jones (26), Brix (7), and others (13) have shown carbon dioxide to be the primary factor limiting growth of higher plants, even though a constant source is available from the atmosphere.

Birge and Juday (5) measured the complete removal of carbon dioxide and severe depletion of bicarbonate and carbonate from soft, medium hard, and hard water lakes in Wisconsin in 1904-1906. They also stressed the importance of bacterial oxidation as a source of carbon dioxide. Pearsall (25), investigating the English Lake District, found higher populations of planktonic diatoms occurring in water containing more $CaCO_3$.

Meyers (24) studied the effect of carbon dioxide and pH on the growth of Chlorella pyrenoidosa. He found that carbon dioxide was growth-limiting, even at low light intensities, and stated that greater growth limitations could be expected at higher light intensities.

Allen (3) found that algae in sewage oxidation ponds were utilizing carbon dioxide instead of organic carbon for growth and that this carbon dioxide was produced by heterotrophic activity.

Wright and Mills (38) and Wright (39) reported a reduction in primary productivity along the Madison River that they correlated with successively lower concentrations of free carbon dioxide in the water. The reduction in

carbon dioxide was attributed to increased photosynthetic activity during the day and to the continual diffusion from the water to the atmosphere.

Kerr et al. (15) demonstrated with laboratory experiments that increasing the amount of carbon dioxide available to an axenic, or "pure", culture of A. nidulans increased its growth rate significantly. This increase occurred when the carbon dioxide was produced by a bacterial culture and also when it was introduced artificially from a gas cylinder.

Kuentzel (17), reviewing much of the recent literature, stressed that the symbiotic relationship between algae and bacteria was an important factor in supplying carbon dioxide to algae. He believed bacterial oxidation to be the major source of carbon dioxide supplied to the algae and that this carbon dioxide is the limiting nutrient in eutrophication processes.

Data from several studies of artificially fertilized ponds indicate the importance of carbon in controlling the growth rate of algae. Thomaston and Zeller (34) reported that additions of inorganic nitrogen and phosphorus did not always result in increased phytoplankton populations. They found growth to be controlled by carbon dioxide availability. Brezonik and Putnam (6) conducted similar studies on two small Florida lakes. They concluded that

additions of phosphates and nitrates alone do not, in all cases, stimulate algae growth.

Kerr et al. (15) concluded from experiments using sterilized pond water as a growth medium that additions of carbon were more important than additions of phosphorus and nitrogen in controlling growth of an axenic culture of A. nidulans.

Abbott (1), conducted artificial fertilization experiments in semi-eutrophic estuarian water within a number of 19-liter carboys. He varied the nitrogen to phosphorus ratio in the water from 10 micromoles per liter of nitrate and one micromole per liter of orthophosphate to 100 micromoles per liter of each. He reported little change in any of the microcosms and concluded that organic components of the estuarine environment must play some role in eutrophication.

Lindemann (20) conducted experiments in order to test the stimulative properties of carbon dioxide on algae grown in flasks. He reported that algal cultures, connected with tubing to flasks containing bacterial cultures, produced denser algal growths than cultures in flasks either open or closed to the atmosphere. Apparently, the carbon dioxide that evolved during bacterial growth was responsible for the increases noted in algal populations.

Lange (18) conducted experiments in which he demonstrated that additions of organic carbon to algal cultures

containing bacteria significantly increased the growth rate of the algae. However, additions of organic carbon to axenic algae cultures did not stimulate growth. He concluded that increased carbon dioxide production by the bacteria was responsible for increased algal growth.

King (16), studying the role of carbon in eutrophication, stated that the main source of inorganic carbon for algal growth is the natural alkalinity system of the water. He concluded that in a natural aquatic environment different species of algae will succeed one another, depending on the amount of available inorganic carbon in the alkalinity system of the water.

In a recent study, Goldman et al. (10) concluded that although carbon may be the limiting nutrient in some special situations (e.g., sewage lagoons and laboratory experiments) it would not normally be limiting in natural environments because it is available in excess at all times in the water. He stated that, in most cases, phosphorus would probably be the logical nutrient to control if one wished to limit algal growth in receiving waters.

Phosphorus, in the past, has generally been regarded as the limiting nutrient in the processes of eutrophication. Even more recently, despite evidence concerning the importance of carbon, phosphorus is still regarded by many as the logical nutrient to remove from wastes before discharge in order to control eutrophication.

In an early study conducted by Birge and Juday (14) in the 1920's, the nitrogen and phosphorus content in 479 lakes in Wisconsin were measured. The average total phosphorus concentration of the lakes was .023 milligrams per liter. The average soluble phosphate was found to be .003 milligrams per liter, while the average organic phosphorus concentration was .020 milligrams per liter. In another study, the overall phosphorus concentration of Lindsley Pond was reported by Hutchinson (12) to be .021 milligrams per liter.

MacKereth (21) stated that measurements of dissolved phosphate in Lake Windermere have shown the phosphate concentration to vary from one milligram per liter to a maximum of two milligrams per liter. He stated that this fluctuation bore no relationship to the size of the population of A. formosa, the dominant algal species in the lake.

In the 1940's Sawyer (30) reviewed studies conducted in 17 Wisconsin lakes, several of which exhibited massive algal blooms attributed primarily to the addition of sewage treatment plant effluents. He concluded from an analysis of data that nuisance conditions can be expected in water when the concentration of inorganic phosphorus equals or exceeds .01 milligrams per liter.

Weiss (37), discussing the relationship between phosphates and eutrophication, emphasized that biological storage, secretion, and reuse of phosphorus by algae are important factors in satisfying nutrient requirements. He also emphasized the importance of the chemical and physical characteristics of the basin in any limnological study of eutrophication.

Kerr et al. (15) conducted experiments using the blue-green algae A. nidulans. They concluded that the extent of phosphorus uptake by this algae is dependent upon its cellular concentration of that element. When the intracellular phosphorus concentrations were low, the amount of uptake was higher than when adequate phosphorus was available inside the cells.

Al Kholy (2) and MacKereth (21) demonstrated uptake and storage by algae in waters containing one microgram per liter or less of phosphorus. This accumulated phosphorus was used to support growth and cell division in the absence of an external supply.

Maloney (22) stated that a synthetic detergent stimulated growth of the unicellular green algae Chlorella pyrenoidosa. He concluded that sodium triphosphate in the detergent was responsible for the growth.

Studies have also been conducted concerning the relationships between phosphorus availability and bacterial

growth. Varma and Stonefield (36), investigating the uptake of P^{32} and Ca^{45} by pure and mixed cultures of bacteria, concluded that there is good correlation between growth of bacteria and uptake of phosphorus. They did say, however, that this uptake capacity varies with species of bacteria.

Rigler (27) added P^{32} to autoclaved lake water and demonstrated that both attached and suspended bacteria utilized inorganic phosphorus during growth. He concluded that the growth of large populations of bacteria must be an important factor in the removal of phosphate from solution.

Hayes and Phillips (11) showed that uptake of phosphorus by bacteria and higher aquatic plants prevented that element from being incorporated into lake sediments. They concluded that bacteria are more efficient than aquatic plants in keeping the phosphorus available in the water column under both aerobic and anaerobic conditions.

Rigler (27), reporting on experiments in which the amount of P^{32} uptake was determined for algal and bacterial fractions of plankton, concluded that bacteria are primarily responsible for the cycling of inorganic phosphate and "might be competing with the algae for the inorganic phosphate."

Research indicates that bacteria contain a larger quantity of phosphorus on a dry weight basis than algae. McKinney (23) reported that bacteria contain 2.5 per cent

phosphorus, and Taylor (33) reported B-strain E. coli contained 2.72 per cent phosphorus. Sanders (15) found that the phosphorus content of planktonic stream bacteria ranged from 1.07 to 2.02 per cent by dry weight and that of attached bacteria ranged from 1.5 per cent to 3.1 per cent phosphorus.

Levin (19) conducted experiments from which he concluded that bacterial uptake of phosphorus is not increased when the concentration of that element in the media is increased. He found that within the range of approximately 5 - 33 milligrams per liter soluble orthophosphate the rates and amount of uptake of phosphorus were independent of concentration.

Kerr et al. (15) conducted experiments using natural heterotrophic populations. She concluded that additions of nitrogen and phosphorus to sterilized pond water stimulated carbon dioxide production by these populations. She also found that nitrogen and phosphorus additions to unsterile water from an infertile farm pond resulted in increased numbers of bacteria, increased amounts of dissolved carbon dioxide, and increased numbers of algae.

Although carbon and phosphorus have received most of the attention in the past, it should be noted that there have been other factors mentioned which have been shown to stimulate or inhibit the growth of algae.

Azad and Borchardt (4) have demonstrated the importance of light intensity as a regulator of algal growth. They also mentioned several other parameters they consider important in affecting the growth rates of algae. Among the significant variables mentioned were: dark-light cycles, temperature, mixing and the ratio of organism mass to water mass. Shapiro et al. (31) have demonstrated that solutions of various salts (e.g. magnesium) can stimulate algal growth.

In summary, the literature indicates the following:

1. There is evidence to support the theories that either carbon or phosphorus can be growth limiting in algal populations.

2. The role of bacteria has been shown to be one of importance in the stimulation of algae growth.

3. There are other factors affecting algal growth rates, among them temperature, mixing, and trace elements.

The relationships between algal and bacterial growth that were mentioned in the literature provided the initial impetus for the research reported herein. The stimulatory properties of phosphorus to bacterial populations were of particular interest, because, if such stimulation could be demonstrated, some inferences could then be made concerning a possible indirect role of phosphorus in stimulating algae growth in aquatic systems.

MATERIALS AND METHODS

The basic experimental procedures used in this research provided for the cultivation of a pure culture of a typical bacterium in a sterile synthetic medium, first in flasks, and later in a continuous-flow culture vessel. Phosphorus concentrations in the medium were varied, and the resultant bacterial growth was evaluated by turbidimetric and respirometric methods. The materials and procedures used are described in this section.

A. Glassware Preparation

All culture vessels used in the experiments were washed thoroughly with detergent (Alconox), then rinsed with distilled water to remove any residual phosphate that might affect final results. Glassware used in phosphate determinations was washed with aqua-regea and then rinsed with distilled water and air dried.

B. Composition and Preparation of Media

The basic composition of the media used is shown in Table I. This medium is basically that described by Davis (8) as M_1B_2 except that sucrose was substituted for glucose. This substitution was made in order to avoid, as much as possible, a brownish-yellow color that developed when the media were autoclaved due to breakdown of the sugar.

TABLE I

COMPOSITION OF MODIFIED M_1B_2 MEDIA

Constituent	Amount (gm/l)
Sodium Citrate; $C_6H_5Na_3O_7 \cdot 2H_2O$	10
Sucrose; $C_{12}H_{22}O_{11}$	10
Calcium Chloride; $CaCl_2 \cdot 2H_2O$	0.10
Magnesium Sulfate; $MgSO_4 \cdot 7H_2O$	0.05
Ferrous Sulfate; $FeSO_4 \cdot 7H_2O$	0.01
Ammonium Nitrate; $(NH_4)NO_3$	6
Disodium Phosphate; $Na_2HPO_4 \cdot 7H_2O$	3.7
Dipotassium Phosphate, anhydrous; K_2HPO_4	2
Phosphate Content 2400 mg/l	

The concentration of phosphate in the media could be varied by the addition of appropriate quantities of solutions of potassium dihydrogen phosphate. A stock solution, containing 0.5 milligrams per milliliter of phosphate was prepared by dissolving 0.7165 grams of anhydrous potassium dihydrogen phosphate in a liter of distilled water. A solution containing .05 milligrams per liter of phosphate was prepared by diluting 100 milliliters of the stock solution to one liter.

In the flask studies 100 milliliter aliquots containing media with various concentrations of phosphate and a predetermined amount of one normal sodium hydroxide (0.4 milliliters per 100 milliliter aliquot) were prepared, dispensed into 250 milliliter Erlenmeyer flasks and sterilized by autoclaving. The sodium hydroxide was added to prevent excessive reduction of pH in the media during autoclaving. This reduction occurred because the amounts of phosphate buffers present in the originally described media were reduced for these studies. In addition, sugars in media normally break down, to some extent, when autoclaved, producing acidic compounds.

For the continuous flow experiments the media were prepared to eight liter quantities. The preparation involved first, dissolving the proper amounts of chemicals into two liters of distilled water. This concentrate was

then poured into the 12 liter Pyrex reservoir and the appropriate amounts of distilled water, sodium hydroxide and phosphate solution were added to give a total of eight liters. The media were autoclaved prior to use.

C. The Test Organism

A culture of Escherichia coli was obtained from the American Type Culture Collection (No. 10536) and was subcultured and maintained on nutrient agar slants. Cell suspensions were prepared by placing a loop of bacteria, taken from a slant stored at 35° centigrade, into 100 milliliters of sterile saline solution containing no phosphate. Inoculation was accomplished with a sterile pipette by the addition of five milliliters of suspension to each continuous culture experiment and one milliliter to each flask culture.

D. Culturing Techniques

The media, containing amounts of phosphate ranging from 2400 milligrams per liter to none at all were prepared as previously described and inoculated with the E. coli suspension. Flask cultures were maintained at 20° centigrade for periods ranging from 48 hours to six days. The continuous culture experiments were maintained, while stirring, at ambient room temperature for periods ranging from 77 to 140 hours. The room temperature usually ranged from 75° to 85° Farenheit.

Continuous Flow Culture. The continuous culture apparatus, shown in Figure 1, consisted of a one liter culture vessel (Bellco Corp.) shown in detail in Figure 2. The vessel was connected by 3/8 inch gum rubber tubing to a 12 liter, round bottom Pyrex media reservoir. A brine tube packed with alternating layers of cotton and glass wool served as an air filter (B, Figure 1) which filtered the air entering the media reservoir while allowing the reservoir to equalize in pressure with the atmosphere as the media were withdrawn. The media were pumped from the reservoir to the culture vessel using a peristaltic-type pump. Flow rates up to two milliliters per minute were produced by using pump motors of varying speeds.

With the exception of the pump, the entire system was sterilized prior to each use. This was accomplished by dismantling the system, wrapping all tubing in aluminum foil and autoclaving the entire system for approximately 45 minutes at 15-18 pounds pressure. Immediately after autoclaving, a clamp was attached to the end of the gum rubber tubing that was to be connected to the culture vessel (Figure 1). The tubing was then cut behind the clamp using a pair of flame sterilized scissors and attached (Figure 2) to the culture vessel. This procedure was followed in order to avoid contamination of the tubing. In addition, all other glass connections in the system were sterilized by flame before being connected.

A. 12 liter Pyrex media jug
B. Air filter
C. 3/8" gum rubber tubing
D. Pump
E. Culture vessel
F. Overflow jug
G. Magnetic stir table

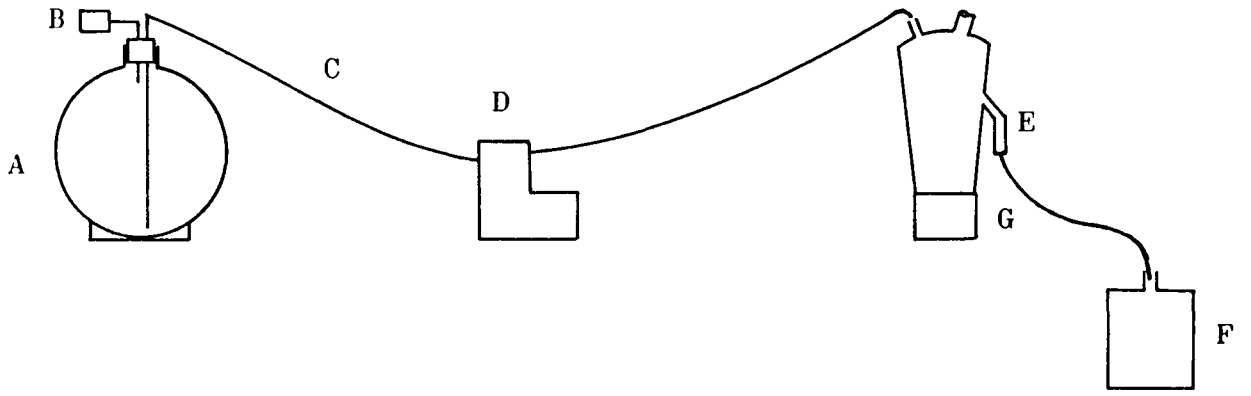

Figure 1. Continuous Culture Apparatus.

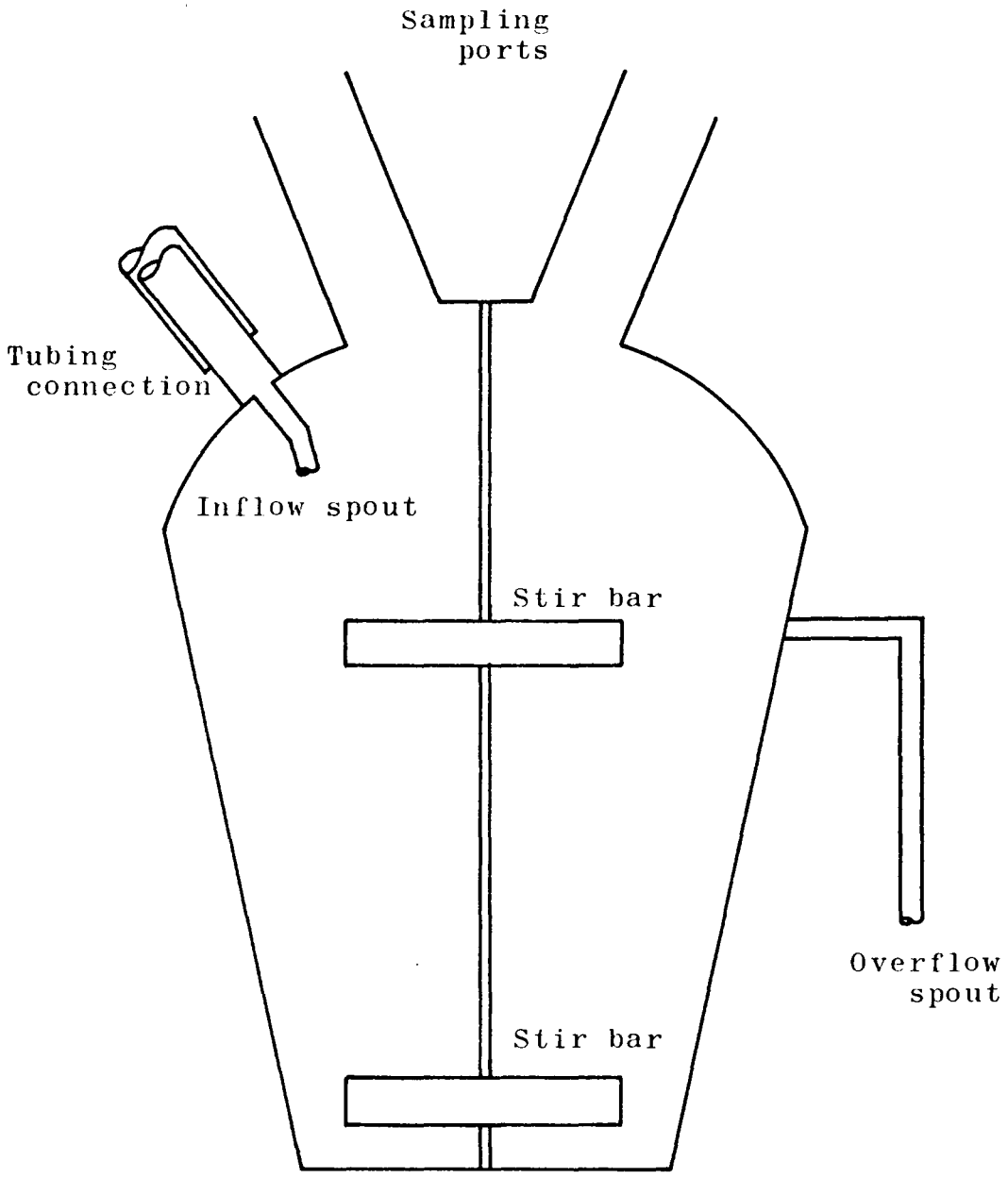

Figure 2. One Liter Bellco Culture Vessel.

Inoculation was accomplished by first allowing approximately 25 milliliters of medium to flow into the culture vessel. Five milliliters of suspension, prepared as previously described, were then added using a sterile pipette. The vessel was then allowed to fill until approximately one-half full, at which time the pump was turned off for 18 to 20 hours to allow growth to occur. The pump was then restarted and allowed to run for the duration of the experiment. In the constant flow growth tests the flow rate was maintained at two milliliters per minute (8.4 hour detention time). However, when investigating the effects of flow rate on growth, the flow could be reduced to one milliliter per minute (16.7 hours detention time) or to .67 milliliters per minute (24.9 hours detention time) as needed.

In all growth tests, samples for turbidity, pH, and phosphate determinations were taken at selected time intervals after the system was inoculated. These intervals ranged from 24 to 94 hours in the constant-flow growth tests. The variable-flow growth tests lasted for longer periods of time; this necessitated taking additional samples as needed. In all tests sampling was begun a short time before the system began to discharge. This was done to detect any changes in the system before and after it had reached a steady state. Turbidity and pH determinations were performed immediately on all samples.

Samples to be used for phosphate analysis were first separated into filtered and unfiltered portions by passage through a .45 micron Millipore filter. One milliliter of "strong acid solution," prepared as described in Standard Methods (32), was then added to each portion to stop any biological activity that was occurring. The samples were then stored in a refrigerator until needed.

E. Growth Assessment

Turbidimetric Methods. Due to the comparative ease and speed with which they can be performed, turbidimetric determinations were chosen as the primary measure of growth in all flask and continuous culture experiments. Measurements were made with the Klett-Summerson colorimeter using a 420 millimicron filter and a four centimeter light path.

Plate Counts. A count was made on each constant-flow growth test after the system reached a steady state condition. In the variable flow tests a count was made after each time the flow rate was changed and the system allowed to re-stabilize. These counts were used as a quantitative check on turbidity measurements. The plate counting procedure used is the standard method described in Standard Methods (32).

Respirometric Methods. Using the Interval method described by Umbreit (35) the Warburg Respirometer was

used to measure oxygen uptake by E. coli. This was done in order to study the effects of phosphate concentration on bacterial respiration. Two types of Warburg experiments were carried out. In the first type, oxygen utilization of E. coli under low phosphate concentrations (.1 milligram per liter) was measured. Excess phosphate was then added to each flask (final concentration: 51 milligrams per liter) and oxygen utilization again measured. Final volume in each flask was 20 milliliters. The inoculum, two milliliters per flask, for this experiment was drawn from the .1 milligram per liter of phosphate, constant flow, growth study. The growth medium used in the flasks was the modified M_1B_2, mentioned previously, with the carbon and phosphate sources removed. The inoculum contained adequate carbon to support growth. In the second type of experiment the oxygen uptake of E. coli was measured under varying phosphate concentrations ranging from .1 to 100 milligrams per liter. The inoculum, two milliliters per flask, for this experiment came from suspensions prepared in saline solution as previously described. The medium used in the flasks was the modified M_1B_2 with varying amounts of phosphate solution added to achieve desired phosphate concentrations. Final volume in each flask was 20 milliliters. In both experiments the thermobarometers contained the M_1B_2 medium with carbon and phosphate sources removed.

Phosphate Determinations. Total, pyrophosphate and orthophate concentrations were determined using the stannous chloride method as outlined in Standard Methods (32). However, these determinations were unsuccessful for two reasons: 1) the breakdown of the sucrose-based media when autoclaved, which produced interfering color and 2) small errors in measurement when high phosphate concentrations required high dilutions in order to get results. Therefore, the results were not included in this thesis.

RESULTS

The effects of several phosphate concentrations and flow rates on the growth of E. coli were investigated by turbidimetric and respirometric methods. The data presented in this section were derived from a number of growth experiments, both in flasks and in continuous cultures. In addition, respiration data were obtained using the Warburg Respirometer.

Preliminary Investigations

A series of experiments was conducted to demonstrate that lack of phosphate in the growth media would limit bacterial growth. Six flasks contained the modified M_1B_2 medium with the prescribed amount of phosphate. Six additional flasks contained the modified M_1B_2 medium without phosphate. One flask in each group was used as a control for that group. After sterilization by autoclaving, the pH of the medium containing phosphate was 7.1 while that of the medium without phosphate was 7.4. The initial turbidity of both media was 20 Klett units. The results of these growth tests are shown in Figure 3. Uninoculated controls exhibited no growth during the test. The results indicated slight growth in the medium containing no phosphate, probably due to the utilization of the intracellular

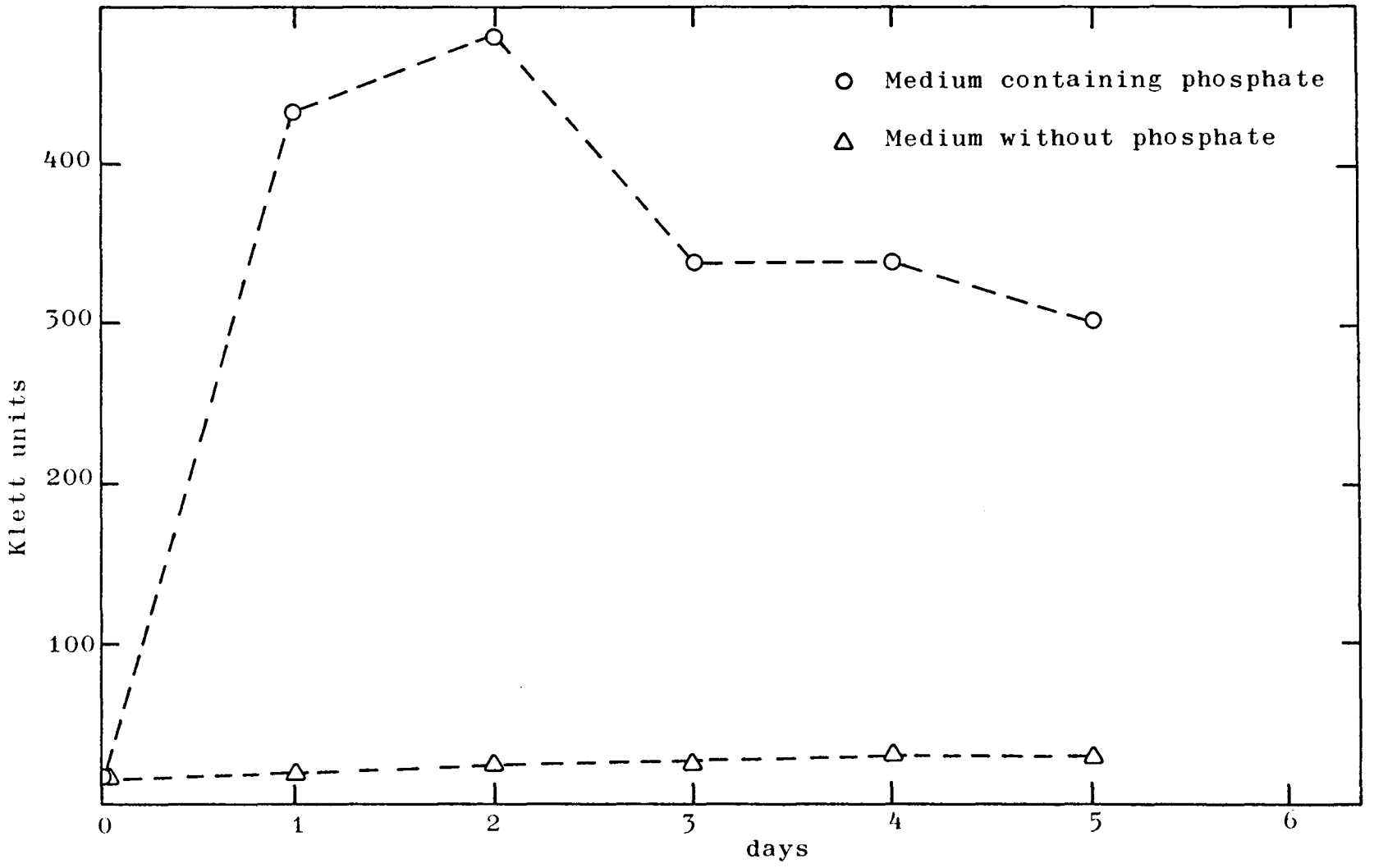

Figure 3. Growth of E. coli in flasks with and without phosphate.

phosphorus compounds present in the inoculum. Growth was significantly greater in the medium containing phosphate, the highest growth level occurring at 48 hours. Turbidity levels then decreased throughout the remainder of the experiment, probably because the accumulation of toxic waste products killed the bacteria. The pH of the medium containing phosphate varied from 6.0 to 6.3 during growth, whereas the pH of the medium without phosphate ranged from 7.4 to 7.6. Each of the control media, which were not inoculated, were pH 7.2 at the end of the experiment. The growth data effectively demonstrated that differences in growth observed in the media were caused by an insufficient concentration of phosphorus in the growth medium.

Having demonstrated that phosphorus is necessary for significant growth of E. coli, it was then necessary to determine the relationship between the growth of E. coli and the concentration of soluble phosphate in solution. This information was needed in order to determine the phosphate concentrations to be used in the continuous culture studies. A series of media, each having a different phosphate concentration ranging from one milligram per liter to 100 milligrams per liter, was inoculated and allowed to remain static at 20° centigrade for 48 hours. At the end of this period the pH and turbidity of each were measured. A control containing no phosphate showed minimal

change throughout the experiment. Results are shown in Figure 4. This experiment indicated that, at least in flasks, relatively little growth occurred at a phosphate concentration of one milligram per liter. These data perhaps indicate that this concentration is at or near the limiting concentration for E. coli. Figure 4 shows that growth varied approximately linearly at phosphate concentrations between one and ten milligrams per liter. Maximum growth occurred at 100 milligrams per liter of phosphate, but the direct relationship between growth and phosphate concentration did not hold. Variation in pH did not appear to limit growth in any of the media. The greatest pH change (7.3 - 5.9) occurred in the medium containing 100 milligrams per liter phosphate that exhibited the greatest growth. The least change in pH (7.3 - 6.3) occurred in the medium containing one milligram per liter of phosphate in which the least growth was observed.

Continuous Culture Studies

In order to investigate the growth of E. coli at various phosphate levels in a steady state condition, continuous culture studies were conducted. The apparati and methodology have been described previously.

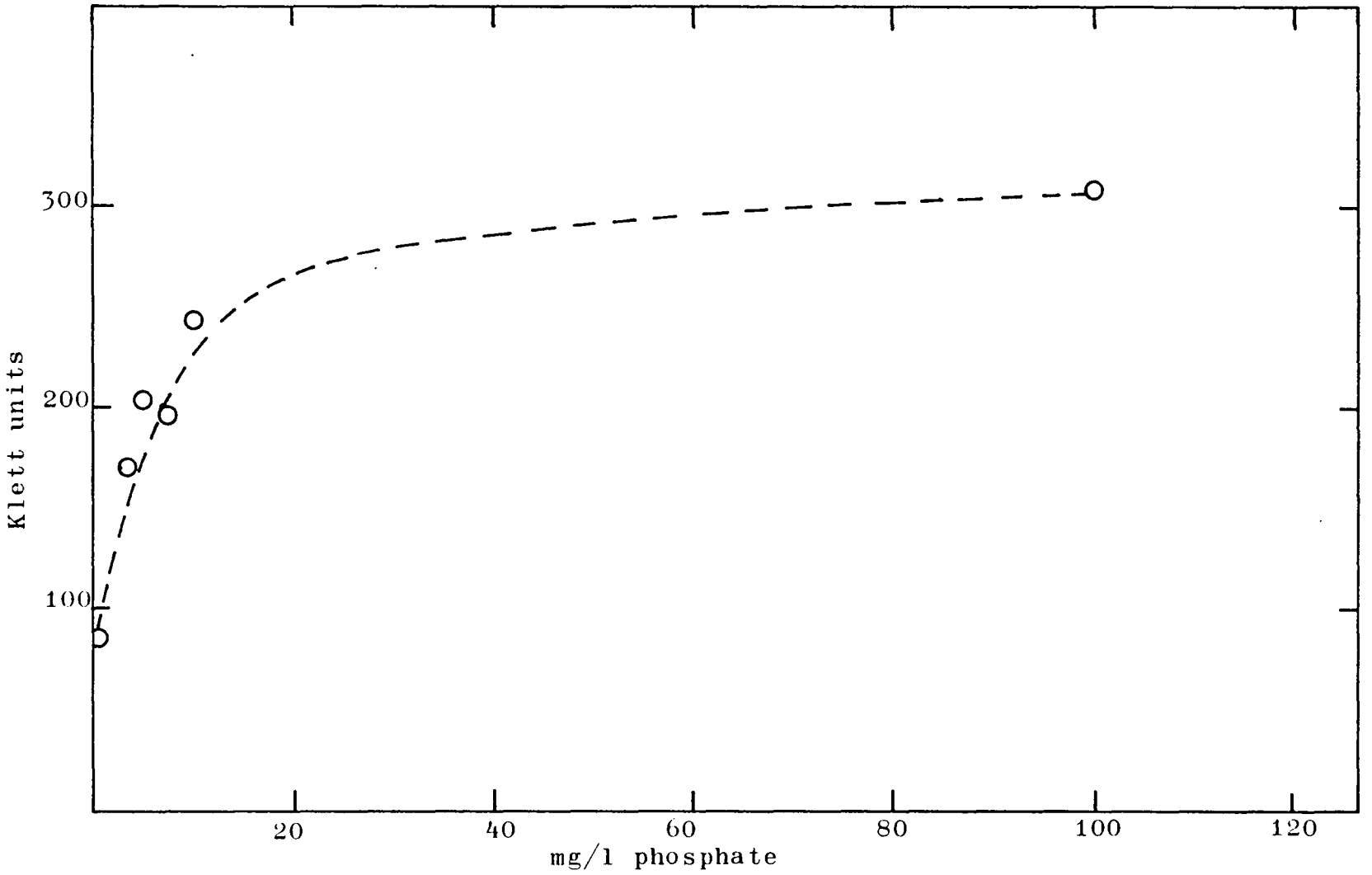

Figure 4. Growth in flasks of E. coli at various phosphate concentrations.

The continuous culture studies were conducted using phosphate concentrations that ranged from zero to a maximum of 2400 milligrams per liter. The flow rate in the system was maintained at two milliliters per minute, resulting in a detention time of 8.4 hours. Each experiment lasted approximately 90 hours. Growth was measured by changes in turbidity expressed as Klett units and checked using plate counts. The pH variations of each experiment were also monitored. The results obtained are shown in Figures 5 and 6 and Table II. From Figure 5 it was noted that the concentration of phosphate in the growth medium had a definite effect on the growth level of E. coli. As the amount of phosphate in the growth medium was decreased the steady state growth level also decreased. Plate counts (Table II), taken at steady state growth levels, verified these results. The pH (Figure 6) also appeared to depend somewhat on the extent of growth but did not appear to limit growth in any of the tests. Of interest in these studies was the initial growth that occurred as the culture vessel filled. It was noted that at higher phosphate concentrations (greater than one milligram per liter) the initial turbidity values were higher than those observed when the system was at steady state. On the other hand, there appeared to be little difference in the two readings at phosphate concentrations of one milligram per liter or less. It was

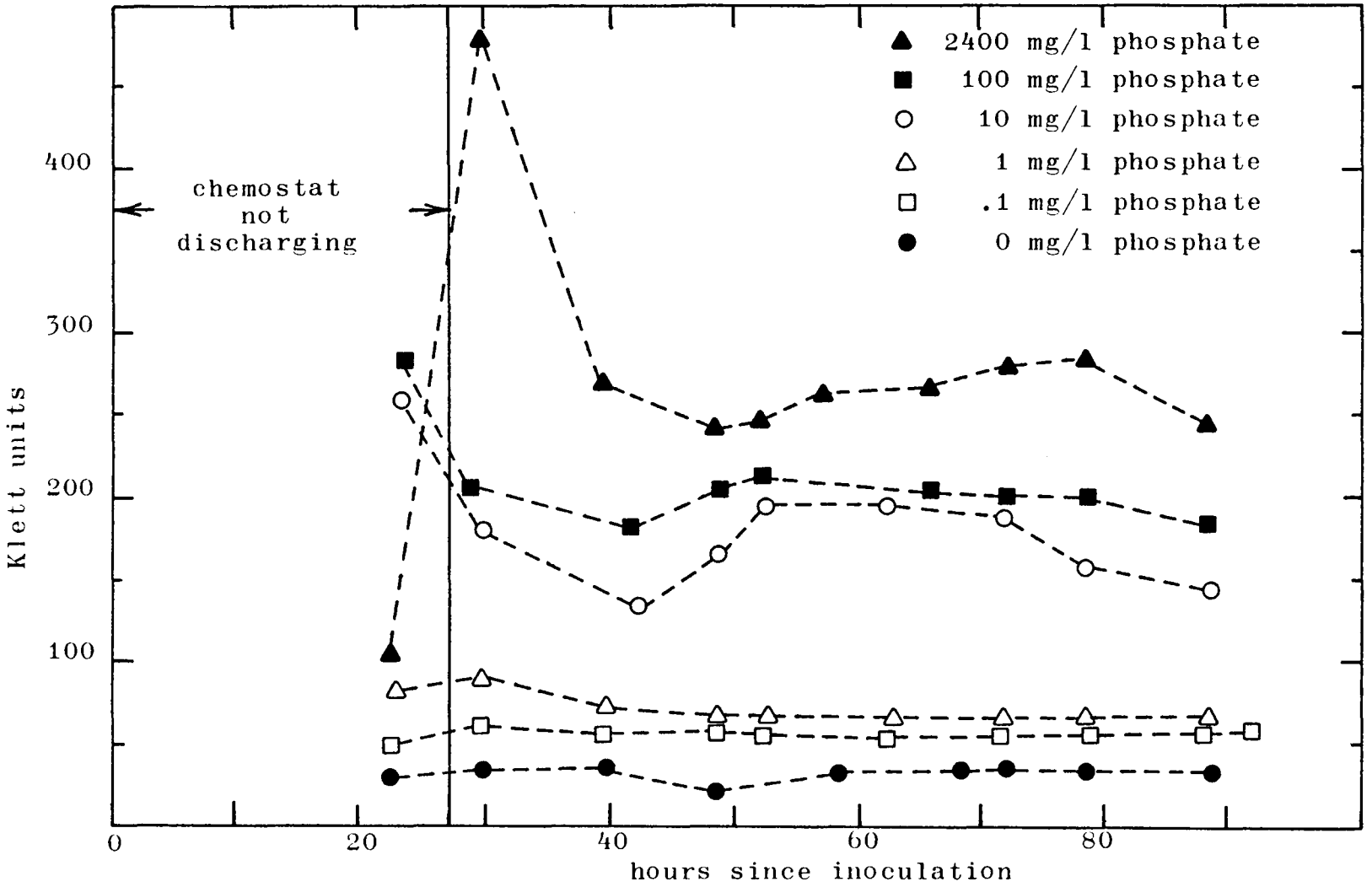

Figure 5. Constant flow (2 ml/min) growth of E. coli at various phosphate concentrations.

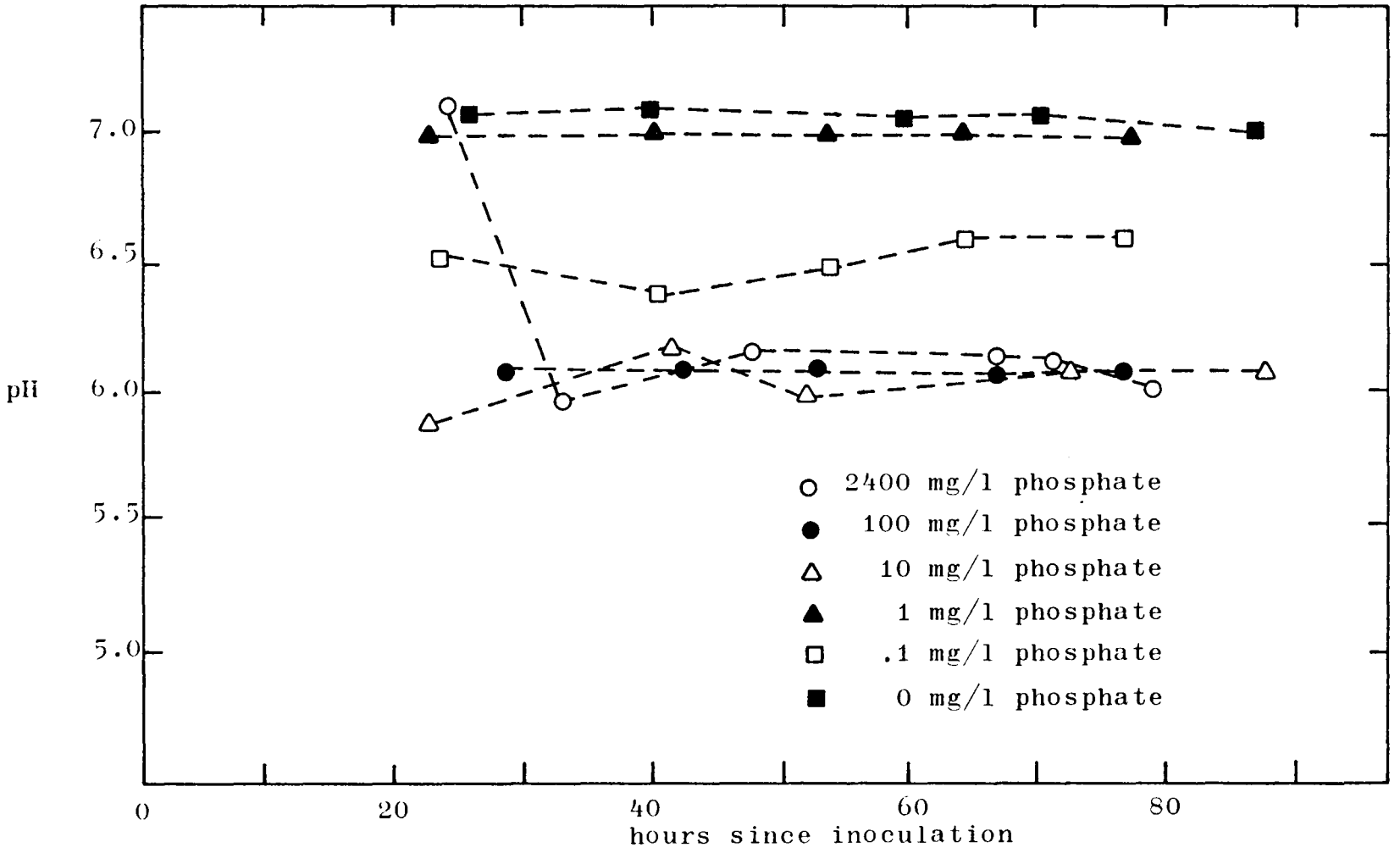

Figure 6. pH measurements from constant flow experiments.

TABLE II

PLATE COUNT RESULTS FOR CONTINUOUS CULTURES

Run No.	Phosphate Conc. mg/l	Inoculum cells/ml	Cells/ml at Indicated Flow Rate 2 ml/min.	1 ml/min.	.67 ml/min.
1	2400	4.32×10^5	1.53×10^7	-	-
2	100	1.42×10^5	1.44×10^7	-	-
3	10	6.63×10^5	1.12×10^7	-	-
4	1	7.61×10^5	1.03×10^7	-	-
5	.1	6.55×10^5	9.91×10^6	-	-
6	0	6.04×10^5	3.92×10^4	-	-
7	.1	6.47×10^5	9.71×10^6	9.91×10^6	9.95×10^6
8	1	6.71×10^5	1.02×10^7	1.03×10^7	1.05×10^7
9	6.25	6.60×10^5	1.05×10^7	1.42×10^7	2.22×10^7

thought that these results might indicate a limiting concentration of phosphate near one milligram per liter. The implications of this are discussed in the following section. Growth that occurred in the experiment having no phosphate in the medium was assumed to have occurred as a result of the use of intracellular phosphorus compounds by the bacteria to support their growth.

Because flow rate (detention time) also was assumed to affect the numbers of bacteria in the vessel at any given time, continuous culture studies were conducted in which the flow rate was changed at intervals. The results obtained (Figures 7 - 9) showed that little change in steady state growth levels occurred at phosphate concentrations of 0.1 and one milligram per liter even though the flow rate was decreased from two to 0.67 milliliters per minute. This caused an increase in detention time from 8.4 hours to 24.9 hours. However, at 6.25 milligrams per liter phosphate a significant increase in the level of growth was noted when the flow rate was reduced from two to one milliliters per minute. The quantity of bacteria present in the growth vessel again increased when the flow rate was reduced further to 0.67 milliliters per minute. Again, plate counts were used to verify these observations (Table II).

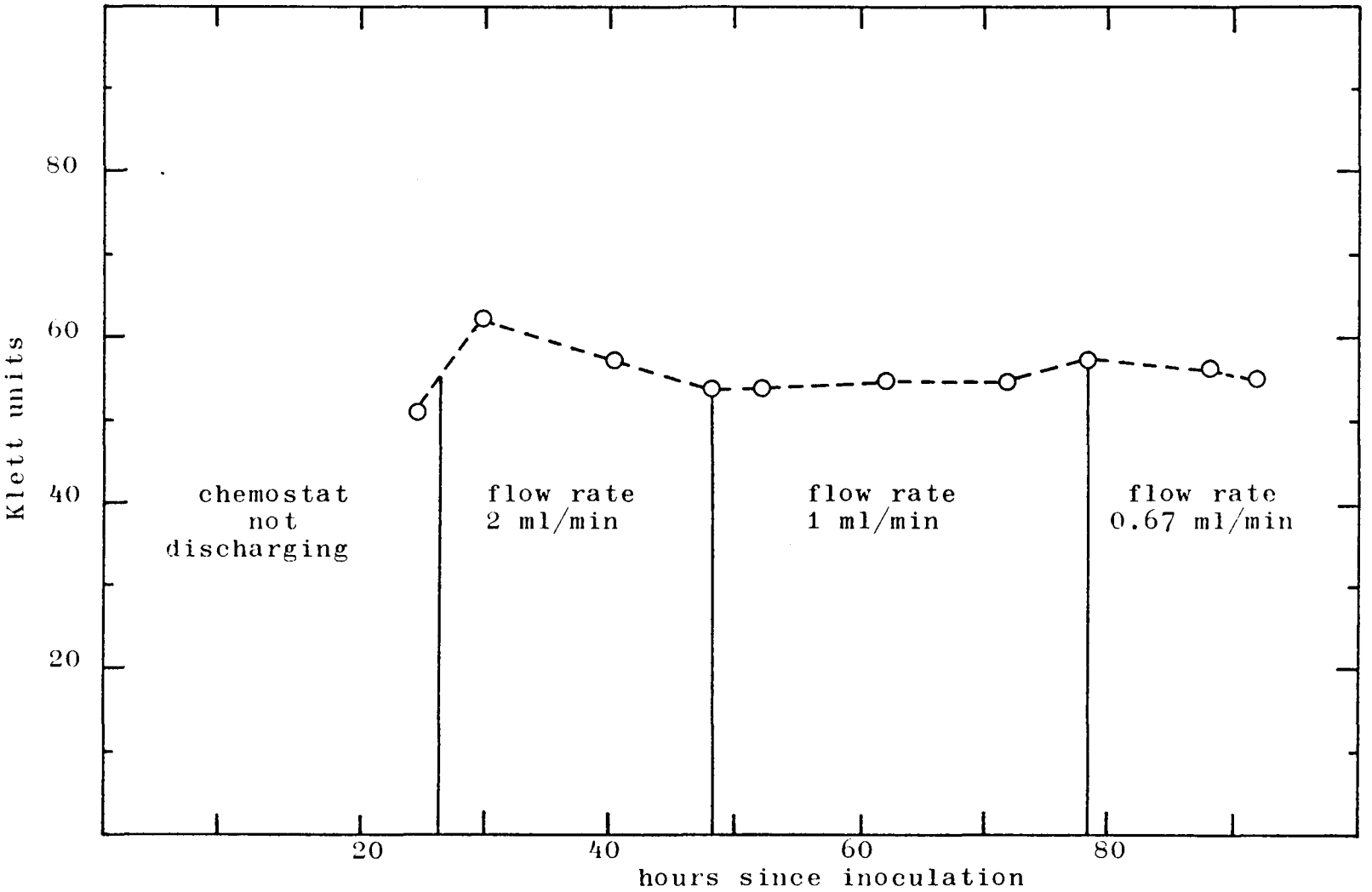

Figure 7. Growth of E. coli at various flow rates and .1 milligram per liter phosphate.

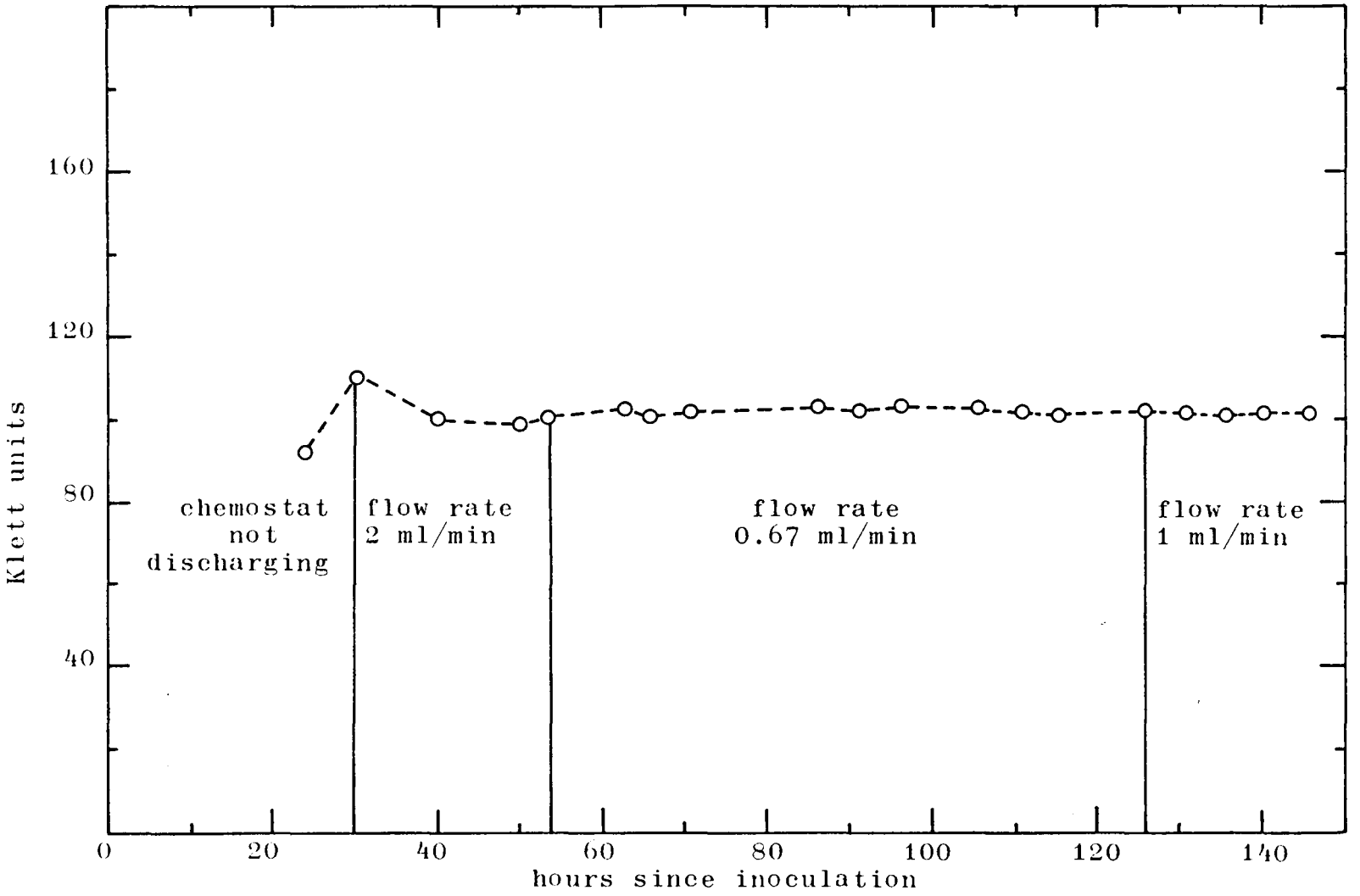

Figure 8. Growth of E. coli at various flow rates and one milligram per liter phosphate.

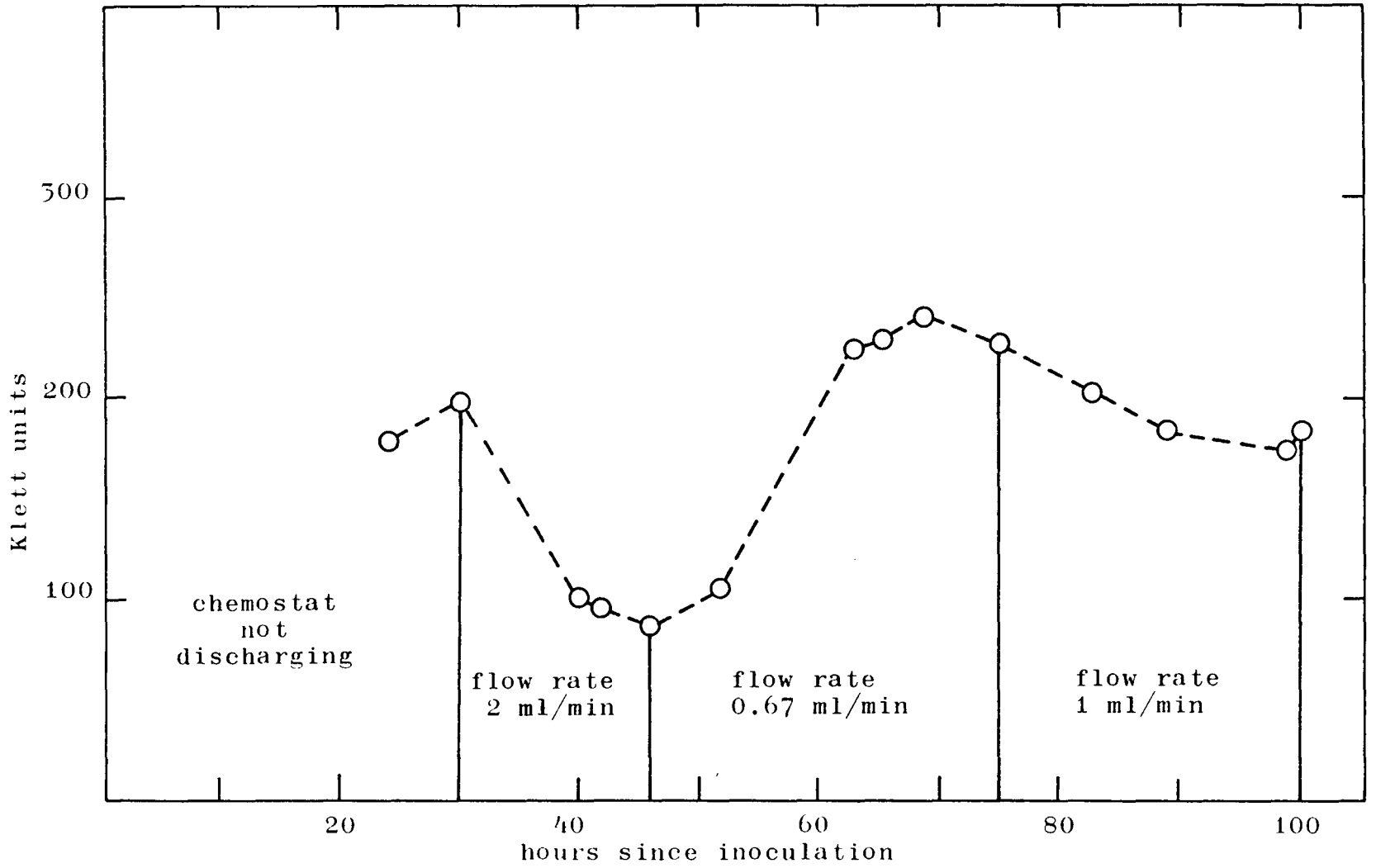

Figure 9. Growth of E. coli at various flow rates and 6.25 milligrams per liter phosphate.

Respiration Studies

In order to study the effects of soluble phosphate concentration on bacterial respiration rates, studies using the Warburg Respirometer were conducted. The basic assumption made in these studies was that the amount of oxygen utilized by the E. coli in a given period of time is equal to the amount of carbon dioxide produced by the bacteria for that same period.

The purpose of the first experiment was to determine the effects of .1, 1.0, 10, and 100 milligrams per liter phosphate concentrations on the respiration rates of E. coli cultures. Results are presented in Figure 10. Significant in this study are the respiration rate changes in the cultures containing 10 and 100 milligrams per liter phosphate as compared to the changes that took place in the cultures containing .1 and 1.0 milligram per liter phosphate.

During the first 36 hours of the experiment, all cultures were respiring at nearly the same rate, about 15 microliters of oxygen per hour. However, at approximately 45 hours the respiration rate in the culture containing 100 milligrams per liter phosphate increased to nearly 570 microliters of oxygen per hour. By the time 50 hours had passed, the respiration rate in the culture containing 10 milligrams per liter phosphate had also

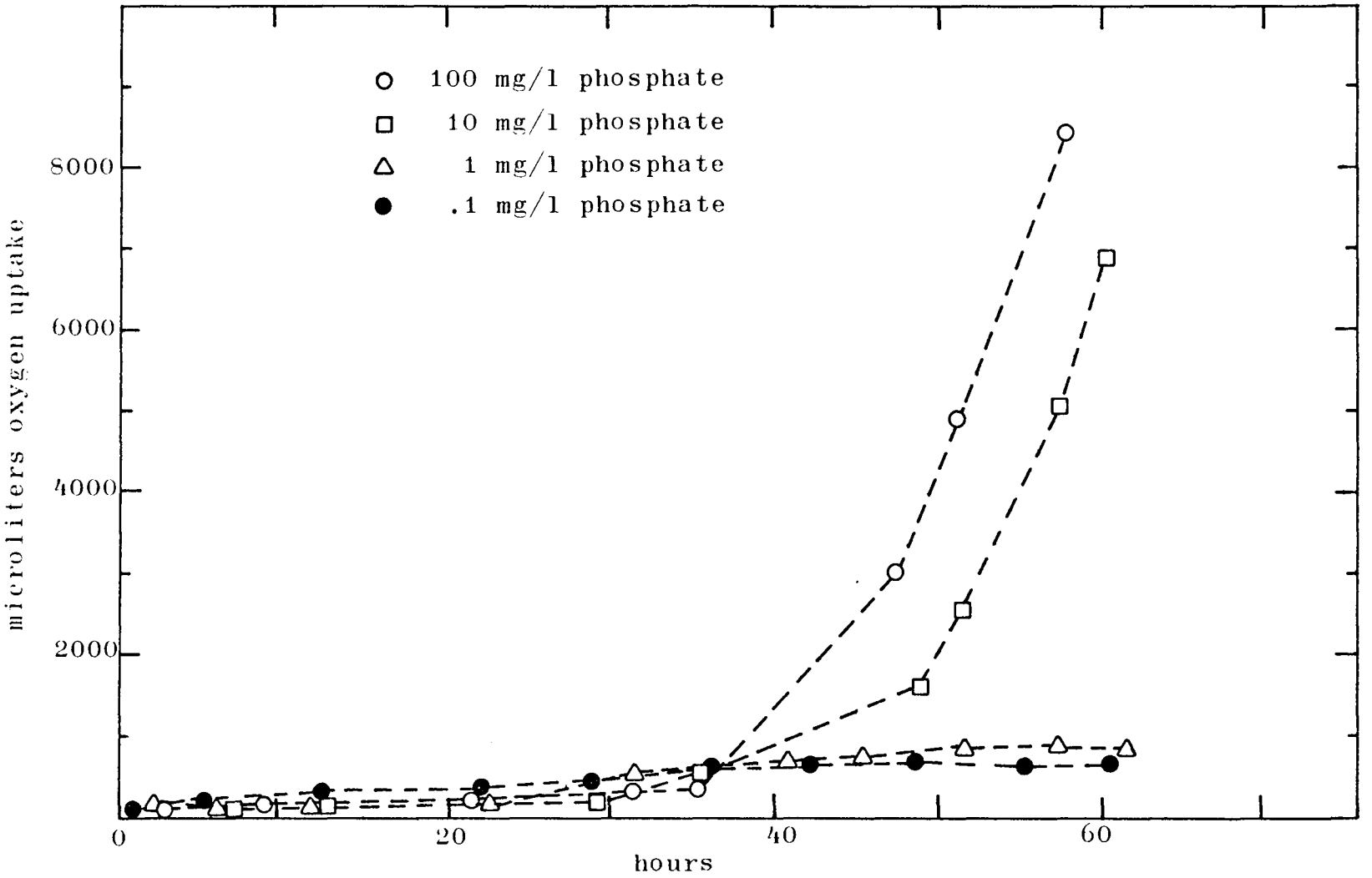

Figure 10. Oxygen utilization by E. coli at various phosphate concentrations.

increased to this same value. Both cultures respired at this rate for the duration of the experiment. During the same period of time the respiration rate of the culture containing one milligram per liter phosphate increased only slightly to 23 microliters per hour of oxygen while that of the culture containing .1 milligram per liter phosphate increased to only 18 microliters of oxygen per hour.

In order to further demonstrate that phosphate can stimulate bacterial respiration rates, a second test was conducted in which the respiration rate of E. coli was measured for 24 hours while growing in medium containing .1 milligram per liter phosphate. At the end of this time an excess of phosphate was added to the cultures and the respiration rate measured again. Results are plotted in Figure 11. The respiration rate of the culture was negligible from 10 to 24 hours. However, upon addition of the excess phosphate the rate of respiration increased immediately to about 12 microliters per hour. A further increase, to 30 microliters per hour was noted 40 hours after addition of the phosphate.

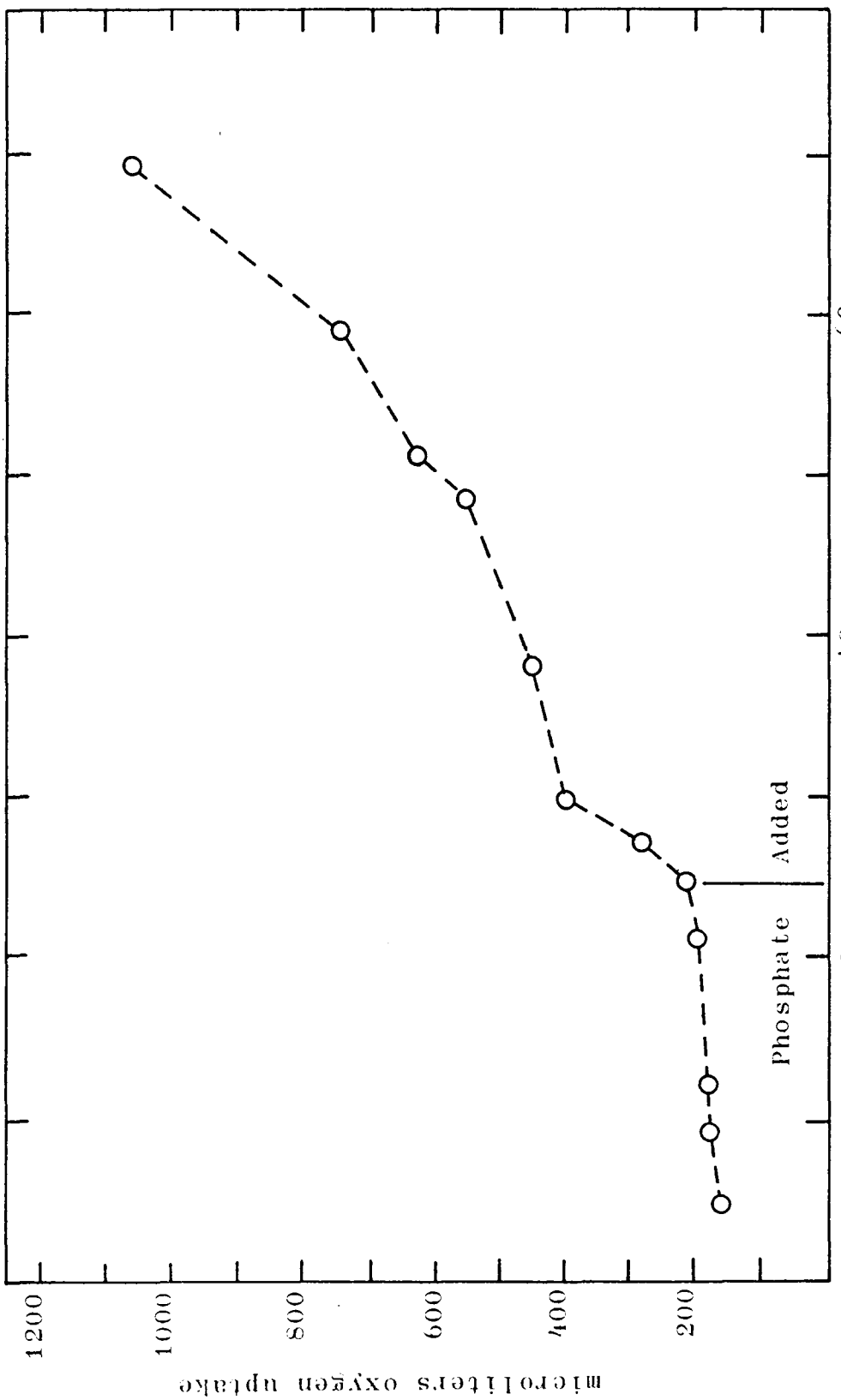

Figure 11. Oxygen uptake of E. coli upon addition of excess phosphate.

DISCUSSION OF RESULTS

It was inferred from the results of this research that, in natural ecosystems where organic nutrients are not limiting, bacterial growth may be restricted by removal of phosphorus from the system. It was also demonstrated that the growth of E. coli is limited at phosphate concentrations of between one and 6.25 milligrams per liter. It was inferred that this limit might apply to other species of aerobic bacteria as well. Accompanying the increased growth of E. coli at high concentrations of phosphate, there occurred a significant increase in oxygen uptake. It was assumed that changes in the rate of carbon dioxide production would be reflected by changes in the rate of oxygen utilization, though no attempt was made to correlate the two. For ease in experimentation, the oxygen uptake rate was monitored rather than the carbon dioxide evolution. Umbreit (35) reported that the carbon dioxide evolved was 80 per cent of the oxygen utilized when fats were metabolized.

It appears that these results may prove to be of significance, particularly in natural systems where inorganic carbon is assumed to limit algal growth. This significance is discussed in the following paragraphs.

Some controversy has arisen in recent years over whether phosphorus or organic carbon should be removed from

surface waters in order to control eutrophication. The controversy is a result of experiments conducted by several researchers (15,18) which have shown that algae growth is increased by an increase in the availability of inorganic carbon. Some researchers (17) have concluded from such experiments that the key to controlling eutrophication is to limit the amount of carbonaceous material that enters a body of water which, in turn, would limit the amount of carbon dioxide produced as a result of bacterial oxidation.

It has been concluded by this investigator that it would be futile to attempt to remove the carbonaceous matter from natural systems. Relatively small amounts of organic matter are required to support a substantial bacterial population. Sanders (29) has mentioned experiments conducted by Heukelekian and Heller (1940) where unlimited growth of E. coli occurred at organic nutrient concentrations of 25 milligrams per liter. Furthermore, attempting to limit organic nutrients in water is complicated by the fact that relatively high concentrations of organic matter arise from sources near bodies of water, especially those receiving wastewater discharges. All of this indicates that it would be difficult, if not impossible, to remove enough organic carbon from carbon-limited surface waters to cause any significant decrease in carbon dioxide production by bacteria. Therefore, it seems that the carbon

dioxide production by bacteria might be better controlled by other methods in systems containing large amounts of organic matter. Such systems would be exemplified by eutrophic lakes and sewage lagoons.

In view of this situation it appears logical that bacterial growth can be controlled if inorganic nutrient removal can be accomplished, as is now being attempted for controlling nuisance algal growths. Phosphorus removal is already considered a feasible procedure to directly control algae growth. Laws, no doubt, will be forthcoming that specify the allowable concentration of phosphorus that wastewater treatment plants may discharge in their effluents. Therefore, it would be advantageous if it could be demonstrated that removal of phosphorus from effluents would not only limit algal growth directly but would limit bacterial growth and, thus, the production of carbon dioxide that might be utilized by algae.

As stated earlier the results presented here indicate that, in fact, the growth of E. coli can be controlled by limiting phosphorus when all other necessary nutrients are present. In addition, E. coli becomes limited in growth at a phosphate concentration between one and 6.25 milligrams per liter. No attempt was made to determine the precise limit. Therefore, it can be inferred that bacterial growth could be controlled in some ecosystems

by the removal of phosphorus. This control in turn, would effect a drop in carbon dioxide production by the bacteria.

Experimental results reported herein have shown that bacteria grown in media containing one milligram per liter phosphate utilize only about 20 microliters of oxygen per hour while those grown at phosphate concentrations of 10 and 100 milligrams per liter utilized 570 microliters per hour. If we assume that carbon dioxide production equals the oxygen utilization, then it appears that increased phosphate concentrations can cause a sizeable increase in carbon dioxide production by bacteria. Logically then, we might restrict the availability of carbon dioxide to algae in aquatic systems containing small quantities of bicarbonate or carbonate by restricting phosphorus availability.

It is also interesting to note that most investigators agree that, when inorganic carbon is not limiting, phosphorus levels must be reduced to .01 milligrams per liter to directly control algal growth. This does not appear technically feasible at this time. However, Figure 10 illustrates that the respiration rate of E. coli increased dramatically when phosphate concentrations were increased above a limiting value of one milligram per liter. Thus, it can be argued that phosphate removal from wastes that will ultimately reach surface waters is advisable even

though current technology does not allow the phosphorus removal to be much more than to one milligram per liter. This would prove significant in systems where organic carbon is not limiting to bacteria and where limited inorganic carbon sources seemed to restrict algal growth. The introduction of high levels of phosphate into this type of system could result indirectly in prolific algal blooms when the carbon dioxide resulting from phosphate-enhanced bacterial metabolism becomes available.

It is important to remember that what has been discussed in this section is an extrapolation of results that were obtained using a continuous flow culture, a synthetic medium and a single bacterium, E. coli. It is by no means conclusive. Other bacterial cultures, pure or mixed, may exhibit different reactions in the same type of system. Also, it must be remembered that there are many biological and chemical processes occurring in a natural system which could influence the results obtained here. The artificial environmental conditions imposed upon this study were not intended to simulate natural systems.

SUMMARY

It would appear that a sizeable amount of carbon dioxide production by bacteria in natural ecosystems can be limited by the removal of excess phosphorus. However, this would only hold true in systems containing sufficient amounts of organic substrate to support a reasonably large heterogeneous population. This phosphorus removal and subsequent decrease in carbon dioxide production by bacteria would help control algae growth in natural systems, especially if the algal growth was limited by the availability of inorganic carbon.

CONCLUSIONS

1. If other nutrients are present in excess it appears that the growth of E. coli can be controlled by controlling the phosphorus concentrations in the growth media.

2. The growth of E. coli appears to be limited at phosphate concentrations of between 1.0 and 6.25 milligrams per liter.

3. E. coli grown at a phosphate concentration of one milligram per liter utilize considerably less oxygen (23 microliters per hour) than E. coli grown at phosphate concentrations of 10 milligrams per liter (570 microliters per hour). This indicates that aerobic bacteria could be expected to produce greater amounts of carbon dioxide when greater concentrations of phosphate are available if these bacteria are not limited by other necessary nutrients.

BIBLIOGRAPHY

1. Abbott, W. "High Levels of Nitrate and Phosphate in Carboy Microcosm Studies" Journal of the Water Pollution Control Federation, XLI (1969), 1748-1751.

2. Al Kholy, A. A. "On the Assimilation of Phosphorus in Chlorella Pyrenoidosa." Physiologia Plantarum, IX (1956), 137-143.

3. Allen, M. B. General Features of Algal Growth in Sewage Oxidation Ponds. State Water Control Board, Sacramento, Calif., Publication No. XIII (1955).

4. Azad, H. S. and Borchardt, J. A. "Variations in Phosphorus Uptake by Algae." Environmental Science and Technology, IV (1970), 737-743.

5. Birge, E. A. and Juday, C. "The Inland Lakes of Wisconsin. The Dissolved Gases of the Water and Their Biological Significance." Wisconsin Geological and Natural History Survey, Bull. No. XXII (1911).

6. Brezonik, P. L. and Putnam, H. D. "Eutrophication: Small Florida Lakes as Models to Study the Process" Proceedings of the 17th Southern Water Resources and Pollution Control Conference. Univ. of North Carolina, Chapel Hill, N. C., (1968) 315.

7. Brix, H. "Influence of Light Intensity at Different Temperatures on Rate of Respiration of Douglas Fir Seedlings." Plant Physiology, XLIII (1968), 389-393.

8. Davis, E. M. Assimilation of Inorganic Nitrogen by Actinomycetes. Unpublished Master's Thesis, Department of Biology, North Texas State University (1962).

9. Fair, G. M.; Geyer, J. C.; and Okun, D. A. Water and Wastewater Engineering, New York: John Wiley and Sons Inc. (1968).

10. Goldman, J. C.; Porcella, D. B.; Middlebrooks, E. J.; and Toerien, D. F. The Effect of Carbon on Algal Growth--Its Relationship to Eutrophication. Utah State University, Logan, Utah (1971).

11. Hayes, F. R. and Phillips, J. E. "Lake Water and Sediment. IV. Radio-Phosphorus Equilibrium with Mud, Plants and Bacteria Under Oxidized and Reduced Conditions." Limnology and Oceanography, III (1958), 459-475.

12. Hutchinson, G. E. "Limnology Studies in Connecticut IV. Mechanism of Intermediary Metabolism in Stratified Lakes." Ecology Monographs, XI (1941), 21-60.

13. Johnson, P. L. and Kelly, J. J. Jr. "Dynamics of Carbon Dioxide and Productivity in an Arctic Biosphere." Ecology, LI (1970) 197-206.

14. Juday, C. and Birge, E. A. "A Second Report on the Phosphorus Content of Wisconsin Lake Waters." Transactions Wisconsin Academy of Science XXVI (1931), 353-382.

15. Kerr, P. C.; Paris, D. F.; and Brockway, D. L. The Interrelation of Carbon and Phosphorus in Regulating Heterotrophic and Autotrophic Populations in Aquatic Ecosystems. U.S. Government Printing Office (1970).

16. King, D. L. "The Role of Carbon in Eutrophication." Journal of the Water Pollution Control Federation XLII (1970), 2035-2049.

17. Kuentzel, L. E. "Bacteria, Carbon Dioxide and Algal Blooms." Journal of the Water Pollution Control Federation, XLI (1969), 1737-1747.

18. Lange, W. "Effect of Carbohydrates on the Symbiotic Growth of Planktonic Blue-Green Algae with Bacteria." Nature CCXV (1967), 1277-1278.

19. Levin, G. V. "Reducing Secondary Effluent Phosphorus Concentration." First Progress Report, Department of Sanitary Engineering and Water Resources, The Johns Hopkins University, Baltimore, Md. (1965).

20. Legge, R. F. and Dingeldein, D. "We Hung Phosphates Without a Fair Trial" Canadian Research and Development (1970) 2-9.

21. MacKereth, F. J. "Phosphorus Utilization of Asterionella formosa Hass" Journal of Experimental Botany IV (1953), 296-313.

22. Maloney, T. E. "Detergent Phosphorus Effect on Algae" Journal of the Water Pollution Control Federation, XXXVIII (1966), 38-45.

23. McKinney, R. E. Microbiology for Sanitary Engineers New York; McGraw-Hill 1962.

24. Meyers, J. "The Growth of Chlorella pyrenoidosa Under Various Culture Conditions." Plant Physiology, XIX (1944), 576-589.

25. Pearsall, W. H. "Phytoplankton in the English Lakes. 2. The Composition of the Phytoplankton in Relation to Dissolved Substances" Journal of Ecology, XX (1932), 241-262.

26. Quinn, E. L. and Jones, C. L. Carbon Dioxide. ACS Monograph Series. New York; Reinhold Publishing Corp. (1936).

27. Rigler, F. H. "A Tracer Study of the Phosphorus Cycle in Lake Water" Ecology XXXVII (1956) 550-562.

28. Ruttner, F. Fundamentals of Limnology. Toronto; Toronto Press (1970).

29. Sanders. W. The Relationship Between the Oxygen Utilization of Heterotrophic Slime Organisms and the Wetted Perimeter Ph.D. Dissertation, Johns Hopkins Univ., Baltimore, Md. (1964).

30. Sawyer, C. N. "Fertilization of Lakes by Agricultural and Urban Drainage" New England Water Works Association LXI (1947) 109-127.

31. Shapiro, J.; Chamberlin, W.; and Barrett, J. "Factors Influencing Phosphate Use by Algae" Advances in Water Pollution Research Edited by S. H. Jenkins. Oxford; Permagon Press, 1969.

32. Standard Methods for the Examination of Water and Wastewater. 12th Edition, New York: American Public Health Association, 1965.

33. Taylor, A. R. "Chemical Analysis of the T_2 Bacteriophage and Its Host Escherichia coli (B^2Strain)." Journal of Biological Chemistry, CLXV (1946), 271-284.

34. Thomaston, W. and Zeller, H. D. "Results of a Six-Year Investigation of Chemical, Soil, and Water Analysis in Lime Treatment in Georgia Fish Ponds." Southeast Association of Game and Fish Commissions. Proceedings of the 15th Annual Conference (1961), 236-245.

35. Umbreit, W. W.; Burris, R. H.; and Stauffer, J. F. Manometric Techniques. Minneapolis, Minn.; Burgess Pub. Co. (1957).

36. Varma, M. M. and Stonefield, D. H. "Uptake of P^{32} and Ca^{45} by Pure and Mixed Cultures of Bacteria" Proceedings of the 21st Purdue Industrial Waste Conference. Lafayette, Indiana (1966) 103-116.

37. Weiss, C. M. "Relation of Phosphates to Eutrophication." Journal of the American Water Works Association LXI (1969), 387-391.

38. Wright, J. C. and Mills, I. K. "Productivity Studies on the Madison River, Yellowstone National Park" Limnology and Oceanography, XII (1967), 568-577.

39. Wright, J. C. "Productivity in Rivers" Proceedings of the Eutrophication-Biostimulation Assessment Workshop, Berkeley, Calif. (1969) 186-206.

The vita has been removed from the scanned document

THE GROWTH OF *E. COLI* AT VARIOUS PHOSPHATE CONCENTRATIONS

by

D. M. Griffin, Jr.

(ABSTRACT)

Accelerated eutrophication is becoming a major problem in relation to surface waters. Controversy has arisen as to its causes in different types of natural systems.

Investigations were made of the stimulatory and limiting effects of phosphate on the bacterium *E. coli* in synthetic media. Growth was measured in batch and continuous flow cultures using both turbidimetric and respirometric techniques.

The results obtained were used to formulate a hypothesis concerning the stimulation of algae by inorganic carbon produced by bacteria and the role this relationship plays in the eutrophic processes that occur in natural systems where organic carbon is not limiting to bacterial populations.